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STUDIES ON IN-VIVO ERYTHROCYTE SENSITIZATION
AND ANAEMIA IN ACUTE SALMONELLA GALLINARUM
INFECTION OF CHICKENS

by

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A thesis submitted for the degree of Doctor of Philosophy
in the University of Edinburgh.

October, 1969.



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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr John Penhale for his constant encouragement and ready assistance. He has been a patient and enthusiastic supervisor and his suggestions and criticisms have been most welcome. I am also deeply indebted to him for many stimulating discussions.

I am also exceedingly grateful to Professor A. Buxton, Head of the Department of Veterinary Pathology in the University of Edinburgh, for the opportunity to work in his Department. He has continued to take a lively interest in the further developments of this project and his continued guidance and helpful counsel have been invaluable.

I wish to express my thanks to members of staff in the Department of Veterinary Pathology for their hospitality in making my stay in the Department a most enjoyable and memorable one. In particular, my thanks are due to Dr G. Fraser for the time spent in preparing the fluorescent photomicrographs and Dr W. Ramsay, of the Department of Biochemistry, for an introduction to radioisotopic techniques and also for permitting me to use his radioactive laboratory facilities.

I most sincerely appreciate the conscientious and meritorious technical assistance rendered throughout by Miss Irene Pow and, at times, by other members of the Technical staff. I am indebted to Messrs. R. Hood and R. James for preparing the photographs and photomicrographs of the figures, and to Miss Mary Buchan for her care and patience in typing the draft and/

and final copies of this thesis.

I am most grateful to the Ministry of Overseas Development for their generous financial assistance which largely financed this project, and to the Department of Veterinary Pathology for the provision of such excellent laboratory accommodation and facilities.

SUMMARY

Factors sensitizing the erythrocytes in-vivo during acute Salmonella gallinarum infection in chickens, the probable mechanism of the associated haemolytic anaemia, and the respective roles played by the phenomena of in-vivo erythrocyte sensitization and the haemolytic episode in the overall pathogenesis of this disease syndrome, were investigated.

Detailed pathological study of the blood during the acute disease showed that, at the time of death, the infected chicken had developed a very severe anaemia and leucocytosis. By relating the reticulocyte response and the absolute haematologic values to the gross and histopathological changes in the bone-marrow, spleen and the liver, it was shown that the anaemia observed was not of the dyshaemopoietic type; it was, however, macrocytic and normochromic.

It was observed that the erythrocytes regularly became sensitized in-vivo during infection and this was subsequently shown to be a common occurring phenomenon in this disease. By examining the biologic, serologic, immunologic, immunochemical and electrophoretic properties of these in-vivo sensitizing factor(s) it was conclusively established that these factors were specific bacterial lipo-polysaccharide and its homologous antibody.

Examination of the patterns of sensitization revealed 4 types of in-vivo erythrocyte sensitization, which were found to be intimately related to the severity and mortality of the haemolytic and the disease syndromes respectively. It was also shown that, chickens in which erythrocyte sensitization was detected, a characteristic binodal erythrocyte fragility curve could be demonstrated.

Techniques were developed for obtaining from infected animals/

v.

destruction. It was demonstrated that the sensitized cells recovered from infected animals may consist of a mixed, heterogeneous cell population of a small minority of 'maximally' sensitized erythrocytes and a larger number of non-sensitized reticulo-cytes.

Normal chicken serum was found to contain a high concentration of heat-stable, non-gamma globulin components which inhibit erythrocyte sensitization by bacterial polysaccharide. These inhibitors, determined to act by either neutralising or altering the polysaccharide in such a way as to prevent its subsequent adsorption by the erythrocyte, were also shown to be significantly decreased in concentration at the peak of the haemolytic episode during acute fowl typhoid.

A moderately severe immuno-haemolytic anaemia could be induced in chickens by single or multiple injections of endotoxin; evidence for the unequivocal participation of specific antibody in the production of this anaemia was obtained by challenging endotoxin-injected chickens with homologous anti-endotoxin antibody.

The significance of the anaemia in the overall pathogenesis of this infection was investigated and it was shown that the anaemia per se did not increase the susceptibility of the chicken to endotoxin. However, prior intravenous administration of in-vivo sensitized, homologous or normal, heterologous erythrocytes markedly decreased the LD₅₀ of the subsequently injected endotoxin. This latter increase in susceptibility to endotoxin : was/

was suggested to be due to a blockaded reticulo-endothelial system, resulting from increased phagocytosis of the foreign and altered homologous erythrocytes.

These results were discussed in relation to the immunological basis of the mechanism of the anaemia and the significance of this anaemia in the overall pathogenesis of this disease syndrome. It was submitted that they were consistent with the hypothesis that the anaemia is the consequence of an immune reaction, involving the specific bacterial polysaccharide and homologous antibody. Furthermore, the anaemia plays an extremely important role in the pathogenesis of the fowl typhoid syndrome.

The possibility that similar phenomena and immunologic mechanisms may be occurring in other acute gram-negative bacterial infections was also stressed.

FOREWORD

The present studies arose as a sequel to earlier observations by Buxton (1959a) that in-vivo modification of avian erythrocytes with bacterial antigen occurred following oral infection of fowls with S.gallinarum, and that chickens fatally infected may develop a severe haemolytic anaemia (Buxton, 1960).

The relationship between these phenomena has been explored and evidence is presented that an immunological mechanism is responsible for the destruction of erythrocytes with consequent anaemia.

The chief objects of study therefore have been the pathogenesis of this anaemia and its significance in the disease syndrome. The value of this particular disease as an ideal experimental model for the study of the mechanism of anaemia of acute microbial infections of both human and animal origin, particularly those caused by gram-negative bacteria, is also stressed.

Reginald K.G. Assoku, October, 1969.

CHAPTER I

GENERAL INTRODUCTION

One of the persisting problems of gram-negative bacterial infection is the exact role the organism plays in the evolution of the disease. Salmonellosis, and fowl typhoid in particular, are no exceptions to this general rule of incomplete knowledge of the pathogenesis of these infections.

Salmonella gallinarum has been known as the causative agent of fowl typhoid ever since Klein (1889) first isolated the organism and recorded the disease in England. According to Beaudette (1930), however, Lemaitre had claimed to have seen such 'typhic epizootics' in France as long ago as 1832 and 1849. In 1895, Moore isolated an organism, which later proved to be the same as Klein's, from an outbreak of an acute disease in chickens which he named "infectious anaemia". Moore (1895) applied the name Bacillus sanguinarium to his organism since he was not aware that it was the same as the one with which Klein had worked. Later, Curtice (1902) studied the disease and named it "fowl typhoid" because it was considered to be analogous to typhoid in man.

Since then many aspects of the disease problem have been investigated with the main objects of assessing and evaluating its economic and public health importance, together with methods of control and chemotherapy (Gordon and Buxton, 1946; Gordeuk, Glantz, Callenbach and Thorp, 1949; Buxton, 1952; Smith, 1955; 1956; Harbourne, 1958; Gordon, Garside and Tucker, 1959). A general review/

review of these aspects of salmonellosis in animals has been published by Buxton (1957).

(I) PATHOGENESIS

General: The paucity of reports on the pathogenesis of S.gallinarum infection in chickens are probably due to the fact that nearly all studies to date have been devoted essentially to aspects of the nature and significance of the "natural resistance factor" to fowl typhoid (Prince and Garren, 1964; 1966; Prince, 1965; 1966; Solomon, 1968).

The first detailed study of the pathogenesis of this infection was by Smith (1955) who found that contrary to the common view that S.gallinarum inhabited, and multiplied, in the alimentary tract all the evidence indicated that any S.gallinarum bacteria found in the intestinal lumen originated from infected foci in the tissues. For example, S.gallinarum was never isolated from the intestinal contents unless it was found in the intestinal wall or in one or more organs which communicated with it. Also, when S.gallinarum bacteria were administered to susceptible chickens by mouth they rapidly disappeared from the faeces and only reappeared when the infection had become generalised. The pathogenesis of this disease was then suggested to closely resemble S.typhimurium infection in mice (mouse typhoid).

It is believed that symptoms and death of individuals infected with Salmonella arise from the action of bacterial endotoxin on host tissues (Dennis and Saigh, 1945; Spink and Anderson, 1954; Taylor, Maltby and Payne, 1959; King and Wood, 1958; Noyes, McInturf and Blahuta, 1959; Okonogi, Fukai, Mitsuhashi, Nagai and Kawakami/

Kawakami, 1959; Grabar, 1959; Wiznitzer, Better, Rachlin, Atkins, Frank and Fine, 1960; Taylor, Wilkins and Payne, 1960; Harvey and Carne, 1960; Buckland, 1960; Cameron, Holtman and Jefferies, 1960; Griesman, Hornick, Carozza and Woodward, 1964; Griesman, Hornick, Wagner, Woodward and Woodward, 1969; Heddlestone, Reberts and Ritchie, 1966). Recognising, therefore, the importance and significance of salmonella lipopolysaccharides as a cause of physiological dysfunction in the host (Thomas, 1954; Atkins, 1960; Landy and Braun, 1964; Zweifach and Janoff, 1965; Nowotny, 1969), Buxton (1960) has suggested that the response of the chicken to preparations of S.gallinarum polysaccharide given under various conditions, could be closely applied to the study of the pathogenesis of fowl typhoid. This author was of the opinion that symptoms and death of chickens were due to the ability of S.gallinarum to multiply rapidly in the host, since it was during the bacteraemic phase that the main symptoms of acute infection could be observed. These included greenish-yellow diarrhoea, loss of weight, inappetance, increased thirst, and shortly before death, the symptoms were typical of a developing anoxia, listlessness, weakness of the legs, cyanosis of the comb and wattles and an apparent state of coma with eyes closed, occasional respiratory gasping, sudden collapse and death. These latter symptoms were all observed within one or two hours before death.

In subsequent studies, Buxton and colleagues (Buxton and Allan, 1963; Buxton and Davies, 1963) assembled suggestive evidence strongly indicating that hypersensitive reactions may be the important factors in the symptomatology and pathogenesis of S.gallinarum/

S.gallinarum infection. The striking similarity between the clinical signs of the acute form of fowl typhoid (Buxton, 1957; 1960) and anaphylactic shock in birds (Makinodan, Wolfe, Goodman and Ruth, 1952; Aronson, Bilstad and Wolfe, 1961) led Buxton and co-workers to suggest that, rather than resulting from toxæmia, it seemed to them that the main manifestations of S.gallinarum infection could be explained to a large extent by the hypersensitive reactions. The results of their experiments showed that during the early stages of infection, circulating leucocytes develop a marked susceptibility to cytophilic antibodies in serum and later to bacterial polysaccharide (Buxton and Allan, 1963). At the time that these cytophilic antibodies are being produced by chickens infected with S.gallinarum, bacterial polysaccharide was rapidly accumulating in the tissues (Buxton and Davies, 1963). Thus, the presence of homologous antibody and bacterial polysaccharide in the tissue of infected birds at death, and the active production of cytophilic antibody prior to death, suggested to them that an antigen-antibody reaction, developing as an anaphylactic type of hypersensitivity (Type I Gell and Coombs (1968) hypersensitivity), may be closely associated with the production of symptoms and death of infected chickens.

The Anaemia in Fowl Typhoid: One of the commonly recorded symptoms of acute S.gallinarum infection is the anaemia (Ward and Gallagher, 1920, Cook and Dearstyne, 1934; Wilson, 1946; 1961; Menzies, 1947; Hungerford, 1951; Buxton, 1957; 1959b; 1960; Hagan and Bruner, 1961; Hall, 1965; Smits, 1966). Surprisingly, however, only few detailed quantitative haematologic studies of this disease have/

have been reported. Ward and Gallagher (1920) had reported that nearly half the total number of erythrocytes had been destroyed by the end of the acute disease. They also observed a massive leucocytosis (from 18.9 to 245×10^3 cells), and they made this interesting observation: "many red blood corpuscles are attacked by leucocytes" at the height of infection.

The first correlated study of the inter-relationships between clinical signs and patho-morphology of blood cells during this disease was by Cook and Dearstyne (1934). They related the development of the disease to changes in the blood cells, and showed that during the initial stages of the disease, the heterophils and monocytes increased in numbers only slightly, whilst the total erythrocyte numbers, haemoglobin and lymphocyte levels fell. Subsequently during the infection, the haemoglobin content progressively decreased and in greater relative proportion than the total circulating erythrocytes. In contrast, the heterophils and monocytes continued to increase dramatically in some of the infected animals until death supervened.

Subsequent reports on anaemia have varied from observations of congestion and cyanosis of comb and wattles (Wilson, 1946; Buxton, 1959^b) and acute anaemia (Smits, 1966) to descriptions of rapid breakdown of red cells leading to paleness of the comb and anaemia (Hungerford, 1951). Hungerford had also observed that "in most severe cases, the comb and wattles were rather dark, and the breathing was accelerated and gasping". Menzies (1947) has reported the comb and wattles to be either pale and bloodless or very dark and congested. He also found that the visible/

visible mucous membranes were unusually very pale and the suggestion was then made that it was the increased destruction of erythrocytes which led to the observed severe anaemia. He estimated that the erythrocytes may be reduced to half the normal number in a few days during infection, whereas the leucocyte numbers were enormously increased. Another interesting observation made by Menzies was that the blood generally appeared thin and watery and did not clot readily. Recently, Hall (1965) made similar observations that the pale comb and wattles generally became more darkly coloured as the disease progressed, and this was regarded as evidence of anaemia and cyanosis.

The first report which gave an indication of the severity and degree of the anaemia was by Buxton (1960), who studied the changes which occurred in haemoglobin levels and fragility of erythrocytes during acute S.gallinarum infection. He observed that chickens fatally infected with fowl typhoid may characteristically develop a severe haemolytic anaemia. During the 3 days preceding death, haemoglobin concentrations showed a rapid decline to approximately one half, or one quarter, of the normal levels. Chickens which survived oral dosage of S.gallinarum showed reduced levels on the eighth day after infection, and these levels began to return to normal on the tenth to fourteenth days in those birds which became clinically normal. However, in contrast, chickens which died a few weeks later, the haemoglobin levels remained at a constant sub-normal level.

Hagan and Bruner (1961) have also described a rapidly developing anaemia and leucocytosis during the acute infection. In addition, they found that one of the main post-mortem lesions/

lesions was a "thin anaemic blood" which was always associated with a splenomegaly.

The mechanism of anaemia of this disease has not been defined, but studies by Buxton (1959a; 1960) have indicated that it may result from an increased destruction of abnormal erythrocytes. Buxton (1960) found that during acute fowl typhoid, circulating erythrocytes developed an abnormal fragility, and also became sensitized (Buxton, 1959a). Sensitization was detected when the erythrocytes were first exposed to anti-S.gallinarum serum and then treated with an anti-chicken whole serum. The resulting positive reactions led Buxton to suggest that the antiglobulin serum was detecting anti-S.gallinarum antibodies which had reacted against in-vivo adsorbed specific bacterial antigen. He also reported that these erythrocyte alterations were demonstrated only when the peak of bacterial infection had developed. From these observations he suggested that the development of the haemolytic anaemia of fowl typhoid is the result of increased ability of the reticuloendothelial system (RES) to increasingly take up both liberated bacterial polysaccharide and in-vivo sensitized erythrocytes.

The Anaemia in Acute Bacterial Infections in General: There have been little attempts in the past to clearly distinguish between the large groups of anaemias associated with acute and chronic bacterial infections; the tendency has usually been to refer to both groups of anaemia as the "anaemia of infection" (Saifi and Vaughan, 1944; Wintrobe, Greenberg and Cartwright, 1946; Cartwright and Wintrobe, 1952; Mitus, 1966).

Although significant anaemia is frequently found associated with chronic disorders, it is a less common event in the course/

course of acute infections (Cartwright and Wintrobe, 1952; Wintrobe, 1967; Editorial, 1967). In these reports, it was stated that when associated with an acute infection, the anaemia usually resulted from accelerated haemolysis. Previously, however, Saifi and Vaughan (1944) had contended that the anaemia of acute infection is dyshaemopoietic, and was due to interference with the synthesis of haemoglobin — the result of maturation arrest in the bone marrow. However, as their results included reticulocytosis, hyperfunction of the bone-marrow and absence of aplasia of erythropoietic tissue, this would suggest that, contrary to their view, the anaemia was not dyshaemopoietic. Later, Brown (1950) in a study of anaemia which complicates certain acute infections and sepsis, put forward evidence to support the thesis that excessive haemolysis frequently plays an important part in the pathogenesis of anaemia of acute bacterial infections.

It is now generally believed that the anaemias associated with gram-negative bacterial infections, especially those of the Enterobacteriaceae, are haemolytic (Dacie, 1967). These bacteria are generally regarded as non-haemolytic, and Dacie has suggested a number of possible mechanisms and phenomena by which an acute infection could damage and rapidly destroy erythrocytes in-vivo. These include T—antigen transformation; the Thomsen-Friendenreich and polyagglutinability phenomena (Reepmaker, 1952; Neter, 1956; Mishra, 1959; Chorpenning and Hayes, 1959; Dausset, Moullec and Bernard, 1959; Springer, 1963); hypertrophy of RES; splenomegaly and splenic hypersequestration (Jandl, Jones and Castle, 1957; Jandl, Jacob and Daland, 1961; Craddock, 1962; Wagner, Iio and Hornick, 1963); auto-immune effect of bacteria on erythrocytes/

cytes (Dacie, 1963; Bedarida, Bernasconi and Polloni, 1961); direct effect of bacterial polysaccharide on erythrocytes — 'indirect bacterogenic agglutination' (Dacie, 1967).

(2) IN-VIVO ERYTHROCYTE SENSITIZATION AND ANAEMIA

A large variety of bacterial antigens readily become attached in-vitro to the surface of erythrocytes of various animal species, as first shown by Keogh, North and Warburton (1948) and later reviewed by Neter (1956). As a result of this sensitization, the erythrocytes can be agglutinated by the homologous bacterial antibodies, and in the presence of complement, haemolysis may occur.

Lipopolysaccharides (endotoxin) extracted from the Salmonellae are readily adsorbed in-vitro on to the surface of erythrocytes, and this has been studied in detail by Spaun (1952) and Fulthorpe (1954). Subsequently, Sieburth (1957; 1958) used the indirect or passive haemagglutination test in studies on avian salmonellosis without realising the potential biological significance of the process of adsorption. In earlier studies, however, Gear (1955 - 1956) had recognised the possible clinical significance of this process in the statement; "It is likely that a similar sensitization of red cells and possibly other tissue cells takes place in-vivo in the course of many of the infective diseases".

The attachment of a bacterial antigen to erythrocytes in-vivo and subsequent destruction of the modified cells by homologous antibodies has been postulated to explain the various haemolytic episodes which have been described in association with certain viral diseases (Moolten, Clark, Glaser, Katz and Miller, 1953; Dacie, 1963), helminth (Koppisch and Oliver-Gonzalez, 1959), and protozoan/

protozoan (Zuckerman, 1964; McGhee, 1965; Cow, 1966), diseases and in penicillin hypersensitivity (Beardwell, 1964; Petz and Fudenberg, 1966; Levine and Redmond, 1967; Croft, Swisher, Gilliland, Bakemeier, Leddy and Weed, 1968)

Moolten and co-workers (1953) had observed in-vivo fixation of virus 'products' on erythrocytes and an associated haemolytic anaemia as a result of blood stream invasion by Newcastle-disease virus. Koppisch and Oliver-Gonzalez (1959), working with extracts of ascaris, were able to sensitize mice erythrocyte in-vivo, and also observed the in-vivo agglutination of such cells. They suggested then that the anaemia of many helminth diseases might have resulted by this mechanism. A similar mechanism was also postulated by McGhee (1965) and Cow (1966) to account for the haemolytic anaemia which sometimes develops during malaria infections. These authors were independently able to produce haemolytic anaemias, in ducklings and mice respectively, by the injection of soluble antigens of plasmodium. These interesting observations have so far not been confirmed by other workers. Similarly certain drugs, especially penicillin, becomes strongly bound to the erythrocyte membrane, and on the appearance of the anti-penicillin antibodies, is quickly destroyed (Levine and Redmond, 1967; Croft et al., 1968; Carstairs, 1968; Worlledge, 1969).

As far as bacterial infections are concerned, the problem is whether in-vivo adsorption of bacterial antigen(s) and subsequent interaction of antibody and antigen(s) at the erythrocyte surface also occurs, and if so, whether this can produce erythrocyte destruction. A few clinical and experimental studies have shown that/

that in-vivo sensitization of erythrocytes may occur during the course of bacterial infections (Buxton, 1959a; Stratton and Renton, 1959; Saito, Konishi, Matsui, Inaba and Okumura, 1961; Young, Gillem and Akeroyd, 1962; Springer and Ansell, 1962; Springer and Horton, 1964; Springer, 1964). However, the occurrence of overt haemolytic anaemia as a direct consequence of sensitization does not seem to have been conclusively proved:

(a) Clinical Studies on In-vivo Sensitization: Skillman, Spurrier, Friedman and Schwartz (1955), claimed that patients with active rheumatic fever had their erythrocytes sensitized in-vivo with streptococcal antigen. Although a number of studies have also indicated that in-vivo sensitization may occur with certain gram-negative bacterial infections (Young et al, 1962; Springer and Ansell, 1962; Springer and Horton, 1964; Springer, 1964) nevertheless, overt haemolysis was not reported to have occurred in any of these cases.

Using the indirect antiglobulin haemagglutination test, Buxton (1959a) had reported that he was able to detect in-vivo sensitization of erythrocytes with bacterial antigen 8 to 10 days following oral infection of chickens with S.gallinarum. He suggested that maximum growth and destruction of S.gallinarum results in the temporary inability of the RES in infected chickens to absorb all available bacterial products, and that a proportion becomes adsorbed on to the surface of circulating erythrocytes.

In 1961, Saito and colleagues recorded that in-vivo erythrocyte sensitization with polysaccharide occurred in 24 out of 67 patients with active pulmonary tuberculosis. Even though they did not report their patients to be also anaemic as well, it is interesting/

interesting to note that severe haemolytic episodes in animal and human patients with active tuberculosis have been described by many workers (Fisher, 1947; Mollison, 1947; Lindeboom, 1950; Sandage, Brandt and Birkeland, 1951; Klimeš and Celer, 1960; Jandl, Jacob and Daland, 1961).

Some interesting observations in man on in-vivo erythrocyte sensitization have been made by Young, Gillem and Akeroyd (1962). They reported that erythrocytes from two infants with gastro-enteritis, caused by E.coli O-127 and O-86 respectively, were shown to have acquired bacterial polysaccharides on their erythrocytes during a relapse phase of their diarrhoea. 'Incomplete' antibodies were said to have adsorbed on to the patients' erythrocytes because the antiglobulin test was positive with each infant's erythrocytes, using anti-human globulin sera. The erythrocytes also agglutinated when first exposed to rabbit anti-E.coli immune sera and then treated with an anti-rabbit globulin serum. From the data, Young and her co-workers suggested that bacterial polysaccharides, some of which have blood group B-like antigens, cross the intestinal mucosa to enter the infants' circulation during attacks of diarrhoea caused by the E.coli pathogens.

Similar findings, where blood group B antigens have been detected in children suffering from severe gastro-enteritis, and in chickens which had been previously shocked or given purgatives prior to infection with the pathogenic E.coli, have also been reported (Springer and Ansell, 1962; Springer and Horton, 1964; Springer, 1964). In some of these cases, Springer and colleagues showed that sensitization was sufficient to produce direct haemagglutination/

haemagglutination of these erythrocytes when exposed to anti-E.coli antiserum. The significance of the above findings is demonstrated by the finding that blood group B-like antigens are identical antigenically to bacterial polysaccharides (Iseki, Onuki and Kashiwaga, 1958; Springer and Ansell, 1962; Springer and Horton, 1964; Springer, Wang, Nichols and Shear, 1966; Springer, 1967.)

This latter finding is particularly important, in view of the observations that patients with severe intestinal disorders, or with extensive intestinal gangrene may acquire de novo blood group B-like antigens (Stratton and Renton, 1959; Marsh, Jenkins and Walther, 1959; Giles, Mourant, Parkin, Horley and Tapson, 1959; Cameron, Graham, Dunsford, Sickles, Macpherson, Cahan, Sanger and Race, 1959; Springer and Horton, 1964), and gram-negative bacteria, particularly those constituting the intestinal flora, possess blood group B specificity (Springer, 1956; Iseki, et al., 1958; Springer, Horton and Forbes, 1959; Springer, Williamson and Brandes, 1961; Springer and Ansell, 1962). In consequence, it has been suggested that these particular acquired B-antigens in the reported cases may therefore be bacterial polysaccharides (Stratton and Renton, 1959; Springer and Ansell; Springer and Horton, 1964).

(b) Experimental Studies on in-vivo Sensitization: In the experimental studies of the phenomenon of in-vivo adsorption of bacterial antigens, two general approaches have been employed:

(1) Investigations in which in-vivo sensitization has been demonstrated by injecting large amounts of bacterial antigens (polysaccharides). For instance, (Boyden, 1953), using a polysaccharide preparation made from tuberculin, showed that erythrocyte sensitization takes place in-vivo in guinea-pigs provided that relatively/

relatively large amounts (10 to 20 mgm) of the polysaccharide were injected. He also demonstrated that the ability to fix these polysaccharide substances was not limited to erythrocytes, but is shared by other blood cells as well. Ceppellini and De Gregorio (1953), however, experimented with rabbits and the Vi-antigen of S.typhimurium, and even though they were unable to sensitize erythrocytes in-vivo by injecting large amounts of the antigen, they did succeed in demonstrating the other aspect of this phenomenon of sensitization. When rabbit erythrocytes were allowed to adsorb the antigen in-vitro and then were re-injected into animals immunized against the Vi-antigen, they found that the injected cells were rapidly removed from the circulation. Such rapidly-destroying cells could also be shown to have been sensitized with the specific immune antigen-antibody complex. Subsequently, De Gregorio (1955) succeeded in sensitizing guinea-pig erythrocytes in-vivo. It was found necessary to inject large quantities of Vi-antigen (10 mgm) intravenously before sensitization could be achieved.

Buxton (1959a) has also obtained similar results in chickens with a polysaccharide extract from S.gallinarum. He reported that the intravenous inoculation into chickens of large doses of crude polysaccharide preparations, derived from a smooth strain of the bacteria, resulted in the adsorption of some of the polysaccharide on the surfaces of circulating erythrocytes. This in-vivo adsorption, which was detectable by an antiglobulin haemagglutination test, lasted for a maximum of approximately 24 hours after inoculation of polysaccharide.

(2) Investigations consisted of injection of bacterial antigen-sensitized erythrocytes into immune and non-immune animals with the object/

object of obtaining evidence of erythrocyte injury and destruction under in-vivo conditions. As stated, Ceppellini and De Gregorio (1953) showed that the Vi-sensitized erythrocytes, which had rapidly been destroyed in Vi-immune rabbits, were however, slowly cleared in non-immune rabbits. These results were recently confirmed by Shumway, Bokkenheuser, Pollock and Neter (1963). These workers, using radiochromium labelled erythrocytes, showed that erythrocytes, sensitized with either polysaccharide extracts (LPS) from either E.coli or S.typhi, were rapidly removed in immune rabbits. Such cells were, however, removed at a moderate rate in non-immune animals. They concluded from their data that the degree of haemolysis and shortening of ^{51}Cr survival depended on the amount of bacterial polysaccharide antigen used for erythrocyte sensitization.

(c) Relationship between in-vivo Sensitization and Anaemia: The biological significance of the phenomenon of in-vivo sensitization, particularly in relation to possible haematologic changes in most of the above clinical and experimental cases, has not been clearly demonstrated.

Of particular interest in this connection is the finding by Julianelle and Reimann (1926) that injection of a crude pneumococcal extract into mice resulted in the development of an anaemia. This has been confirmed recently by Shumway and Pollock (1965), who showed that intravenous administration of an extract of pneumococcus into rabbits results in spherocytosis, increased erythrocyte osmotic fragility and intra-vascular haemolysis. Shumway (1958) had earlier reported that rabbits with acute pneumococcal infection develop a spherocytic haemolytic anaemia.

The/

The question also arises as to whether this phenomenon of in-vivo sensitization contributes to the sludging of blood (that is, intravascular red cell agglutination) observed in certain types of immuno-haemolytic anaemias and other haematologic diseases (Wasastjerna, Dameshek and Kominos, 1954). In this connection, attention should be called to the reports of Nungester and Klein (1937) and Youngner and Nungester (1944), who showed that pneumococcus polysaccharide affects the red blood cells in such a way as to enhance their sedimentation rates in-vitro and to alter their flow rates in-vivo.

A highly significant contribution to the study of effect of intravenously administered endotoxin on the sensitization, survival and rate of destruction of the erythrocytes has been the report of Ho and Kass (1958). They showed that normal rabbits develop a mild haemolytic anaemia after a single intravenous injection of S.typhi endotoxin. A more marked anaemia develops in immunized rabbits which were subjected to repeated inoculations of endotoxin. They also observed that haemolysis occurred in some rabbits during the second week after endotoxin injection. Their failure to detect any in-vivo sensitization of the erythrocytes led them to ascribe this haemolytic reaction to an as yet unexplained activation of the RES.

Buxton (1960) had reported that chickens fatally infected with S.gallinarum may develop a severe haemolytic anaemia. He had also previously observed that during infection the erythrocytes may become sensitized in-vivo with a bacterial product, probably acquired from the infecting organism (Buxton, 1959a).

From/

From these results, Buxton then suggested that the development of the haemolytic anaemia is the result of increased activity of the RES and that during the development of this disease the RES becomes stimulated and therefore is able to increasingly take up liberated bacterial polysaccharide, including the sensitized erythrocytes.

The occurrence of abnormalities of erythrocytes, haemolytic episodes and positive direct Coombs tests (DCT) with the enterobacterial infections in man and animals has not been generally stressed. Nevertheless, there are several recorded observations on these features of these diseases, especially diseases of the Salmonellae. For instance, a haemolytic episode is a well known, although uncommon, complication of typhoid fever in man (Flandin, Bernard and Mallarmé, 1935; Berman, Braun and Rachmilewitz, 1945; Shaw, 1951; Gordon-Smith, 1951; Retief and Hofmeyr, 1965). In 1945, Berman and associates had concluded from their studies that haemolysis, rather than haemorrhage, was the more likely cause of the mild to severe anaemia associated with the high reticulocyte counts of 4.6 to 10 per cent which had developed, usually in the second or third week of illness, in 9 out of a series of 152 typhoid patients.

Evidence for the occurrence of indisputable haemolysis, that is, anaemia accompanied by haemoglobinuria, was reported by Shaw (1951), Gordon-Smith (1951), McFadzean and Choa (1953) and Ruggieri (1961). Significantly, McFadzean and Choa reported that the haemolytic episode occurred only in cases with typhoid, and there was no such haemolysis in patients with paratyphoid A, B and C. Another interesting observation they made was that a large proportion of their typhoid patients had shown a positive direct or/

or indirect Coombs tests.

Similar observations in typhoid patients whose erythrocytes gave positive DCT have also been reported by Grobbelaar (1958). He described 3 human patients with acute typhoid in whom there was suggestive evidence of haemolysis, and obtained positive DCT with 30 out of the 100 blood samples taken from these typhoid patients. He considered that in South Africa typhoid was second only to the malignant reticuloses as a cause of symptomatic haemolytic anaemia in man.

A further interesting account has, more recently, also been reported from South Africa. Retief and Hofmeyr (1965) described the occurrence of severe anaemia and haemoglobinuria as the dominant clinical finding in a febrile male patient. The DCT was negative, but S.typhi was isolated 3 times by blood culture. They observed that both the haemolysis and the pyrexia in their patient subsided within a few days of starting treatment with Chloromycetin. They also found that 50 per cent of the reported instances of acute typhoid haemolysis in the literature, always occurred during the first week of illness when bacteraemia was known to be maximal. Retief and Hofmeyr, while stating that too few authentic cases of acute haemolytic anaemia had been reported in typhoid patients, concluded nevertheless, that the association is possibly more common than is generally thought, and more of such cases would have been forthcoming if they had been looked for in the first week, at the time of bacteraemia.

Other non-typhoid salmonella infections in humans with complicating haemolytic anaemias have also been published. Thus, Davidson and Fullerton (1938) reported having observed an acute/

acute haemolytic anaemia in a patient infected with S.dublin, and recently, Dacie (1967) had diagnosed an acute haemolytic anaemia in a boy, a week or so following infection with S.aertrycke.

The mechanism of these haemolytic episodes, as reported, is not clear, even though McFadzean and Choa (1953) had suggested an underlying auto-immune aetiology, possibly associated with the lymphoid hyperplasia of typhoid fever.

Other enterobacterial infections in man and animals with a clear demonstration of increased erythrocyte destruction or acute anaemia, but not reported to have any in-vivo sensitization, have been reported by numerous authors. For instance, Horowitz, Javid and Spaet (1960) had described the occurrence of anaemia in a small girl who was suffering from an E.coli infection. The haemolysis subsided when the infection responded to treatment with Chloramphenicol. Similar observations on the occurrence of intra-vascular haemolysis in patients with Vibrio cholerae infection were reported in 1954 by De, Sengupta and Chanda. The complication of bovine brucellosis with acute anaemia has also been long recognised, and this has been the basis of a series of reports by Bell and Irwin (1938), Calder, Steen and Baker (1939) and Ferguson, Irwin and Beach (1945).

The occurrence of haemolytic anaemia in other gram-negative bacterial septicaemias, other than Salmonella infection, had also been described by many authors (Brown, Hayward, Powell and Witts, 1944; Pappenheimer et al, 1945; Finch et al, 1949; Brown, 1950; Kleeman and Epstein, 1957; Rappaport, Tatter, Coeur-Barron and Hjort, 1964). In patients acutely ill with gram-negative bacterial/

invasion of the blood stream, some observers are of the opinion that the survival time of the erythrocytes is markedly decreased (Finch et al., 1949; Brown, 1950) and has also been suggested that the release of endotoxin during infection may play a significant role in the pathogenesis of haemolytic anaemias complicating these bacteraemias (Kleeman and Epstein, 1957), although in-vivo erythrocyte sensitization has not been reported to occur in these infections.

In 1948, Middlebrook and Dubos had reported that a mycobacterial component of polysaccharide nature could sensitize erythrocytes and, in the presence of antiserum, haemagglutination took place. These phenomena of erythrocyte sensitization and haemagglutination seem to be analogous to those observed with the polysaccharide component of gram-negative bacteria (Stetson, 1955; Braude, 1959; Sell and Braude, 1961; Tsumita and Ohashi, 1964). In this connection, it has already been mentioned that Saito and colleagues (1961) reported that in-vivo erythrocyte sensitization occurs with patients with active pulmonary tuberculosis, and the possibility that patients with overt tuberculosis may be prone to develop haemolytic anaemia was suggested by Fisher (1947), after reviewing a number of miscellaneous cases of acquired haemolytic anaemia. In addition, human patients with active tuberculosis in which severe haemolytic anaemia has been found to occur, are described by Mollison (1947); Lindeboom (1950) and Jandl, Jacob and Daland (1961). In animals too, Sandage, Brandt and Birkeland (1951) showed that the intravenous injection of small quantities of mycobacterial polysaccharide into rabbits with active tuberculosis resulted/

resulted in increased destruction of erythrocytes. Similar changes in total erythrocyte counts, but different in degree, occurred in rabbits during the course of ultimately fatal tuberculous infection without the injection of homologous polysaccharide. From the available data, these authors suggested that such effects were due to liberation of polysaccharide from the foci of infection, and the possibility that destruction of the erythrocytes was a direct consequence of this activity was also discussed. Chickens with active tuberculosis have also been examined for any characteristic changes in the blood picture by Klimes and Celer (1960) and it was found that there was a reduction in the total number of erythrocytes. Similar results were also obtained from chickens experimentally infected with tuberculosis.

OBJECTS OF THE STUDIES

It is evident from the foregoing review that little is known of the pathogenesis of anaemia of acute bacterial infections, including the haemolytic episode observed by several workers during acute human and fowl typhoids. It was therefore considered that an investigation of this problem, using fowl typhoid as the experimental model, would be of value in the elucidation of the mechanism and significance of the anaemia of bacterial infections, especially those caused by gram-negative bacteria.

Fowl typhoid appeared to be a particularly suitable disease model for this purpose for a number of reasons:

(a) The fact that both the phenomenon of in-vivo erythrocyte sensitization and the clinical anaemia had already been demonstrated in/

in this infection (Buxton, 1959a; 1960).

(b) The ease of establishing fowl typhoid infection and low infectivity for other species, including man.

(c) The well-known refractoriness to endotoxin toxicity by the chicken (Smith and Thomas, 1956; Heilman and Bart, 1964; Truscott and Inniss, 1967).

(d) Availability and ease of handling make chicken an ideal laboratory animal.

The present investigations are divided into 10 chapters. Chapter I has been concerned with an introductory review of relevant literature; this has been limited to reports on erythrocyte sensitization and anaemia of gram-negative bacterial infections, and the mechanisms which are of general importance for understanding the basic mechanism of fowl typhoid.

As a preliminary basis for these studies it was considered that the main haematological changes occurring during S.gallinarum infection in chickens should be fully established and defined, particularly in nature and extent. Accordingly, the second chapter is devoted to haematologic investigation of the disease. The significance of the erythrocyte changes in relation to the immunological basis of the mechanism of the anaemia is considered in Chapter III, and the possibility that the in-vivo erythrocyte sensitizing factor may be bacterial lipo-polysaccharide is examined in the next Chapter (IV). Direct evidence to elucidate the type and mechanism of this anaemia was obtained by radioisotopic studies, and the results are recorded in Chapter V. Chapter VI contains evidence which sought to establish that the in-vivo/

in-vivo sensitized erythrocytes, recovered from infected animals, consist of a mixed, heterogeneous cell population with respect to the degree of sensitization. Chapter VII is devoted to studies involving the responses of the chicken to large doses of S.gallinarum endotoxin in which attempts are made to experimentally induce both in-vivo sensitization of the erythrocytes and a haemolytic episode in this species.

The significance of the anaemia, and the probable roles played by both the phenomenon of erythrophagocytosis by the stationary (fixed) macrophages of the reticulo-endothelial system and the bacterial products (endotoxin) in the overall pathogenesis of the disease syndrome, are examined and evaluated in Chapter VIII. Chapter IX contains studies on the probable mechanism of action of serum 'inhibitors' of erythrocyte sensitization in-vitro; preliminary observations are also made on the changes in these 'inhibitors' during acute S.gallinarum infection.

The last Chapter (X) is devoted to the general discussion and evaluation of the results, together with conclusions.

CHAPTER IIHAEMATOLOGICAL CHANGES DURING ACUTE INFECTIONINTRODUCTION

During the course of acute fowl typhoid various clinical signs, indicative of anaemia, have been frequently recorded by numerous workers (Wilson, 1946; Menzies, 1947; Hungerford, 1951; Buxton, 1957; 1959; Hagan and Bruner, 1961; Hall, 1965; Smits, 1966), but surprisingly, comprehensive haematologic studies on this disease have been few. The only recorded haematologic studies are by Ward and Gallagher (1920), Cook and Dearstyne (1934) and Buxton (1960), and none of these include detailed quantitative studies to define the extent of the haematologic changes during the acute infection. For instance, Ward and Gallagher (1920) only reported the destruction of 50 per cent of the total circulating erythrocyte population, and also indicated that erythrophagocytosis by leucocytes may be taking place in the circulation. The significance of the anaemia in the overall pathogenesis, and the nature of the anaemia were not however, considered.

The study by Cook and Dearstyne (1934) was concerned with the relationship between clinical signs and the pathomorphology of blood cells during the acute infection, and no accurate definition or degree of the anaemia was given.

The first and only report which gave an indication of/

of the severity and degree of the anaemia was by Buxton (1960), who studied the changes which occurred in haemoglobin levels and erythrocyte fragility.

As a preliminary step in the investigation of the pathogenesis of the haemolytic episodes associated with bacterial infections, particularly fowl typhoid, it was considered that studies should be undertaken with the following objectives:

- (1) A detailed study of the pathological changes in the blood to establish the extent of the anaemia and to obtain an indication of its possible significance in the disease.
- (2) Definition of the type and character of this anaemia which may throw light on the aetiological mechanism.
- (3) Preliminary study of in-vivo sensitization of erythrocytes with the intention of confirming the original observation of Buxton (1959a) and to provide a basis for more detailed studies in later chapters.

MATERIALS AND METHODS

Chickens: Adult, outbred female chickens ('Hybrid 66') of approximately 6 months of age, and salmonella-free, were obtained from a large commercial hatchery (Chunky Chick Ltd.) to ensure continuity and uniformity. They were maintained on a standard mash and grain diet, and fed ad libitum.

Infected and control chickens were paired, tagged and kept in separate rooms.

Inoculum: A virulent, smooth strain of S.gallinarum ("strain 938") typed at the commencement of these studies, was used. This strain of/

of Salmonella, which was used throughout these investigations, was preserved by freeze-drying. When required for use, it was sub-cultured on to MacConkey agar plates, and after incubation at 37°C, a smooth colony was transferred to nutrient digest broth and the culture was used after overnight incubation.

Infection: Preliminary infection experiments with this strain of salmonella had shown that to obtain consistently reproducible 100 per cent mortality in less than 7 days, the intramuscular route of infection with an inoculum of 2 mls. of overnight broth culture per animal, was the ideal procedure. Oral dosing of the chickens gave inconsistent and variable rate of mortality. With the intramuscular route of infection there was neither need to pre-starve the animals before injection, nor to administer an alkali with the inoculum as advocated by Smith (1955).

Control chickens received sterile broth digest by the same route.

Sampling Procedure: Blood samples were obtained from the wing vein using E.D.T.A. as the anticoagulant. The probable error in accuracy of the haematological values brought about by continuous bleeding in chickens has been stressed by Cohen (1967). Therefore, in studying blood trends through all the stages of the acute disease, it was advisable to conduct this study on the basis of chickens bled once daily for the first 2 days after infection, and then twice daily for the next 2 to 4 days. The minimum amount of blood to be taken was one to 2 ml. at each sampling, and extreme care was taken to prevent unnecessary blood loss.

Blood samples, collected in Sequestrene (E.D.T.A.) tubes/

tubes (Stynes Laboratories), were gently mixed to prevent clotting and immediately used.

Measurement of Haematocrit: The haematocrit value of each blood sample was determined by the micro-haematocrit method described by Cohen (1967) for avian red blood cells. The method ensures adequate packing of cells and leaves little or no trapped plasma among the packed cells. This is probably the main cause of the variability in the published data in avian haematocrits.

Sealed micro-capillary tubes, containing the blood samples, were centrifuged in a Hawksley Micro-haematocrit centrifuge at 10,000 r.p.m. for 8 minutes. The haematocrit value was then measured directly on a reader. Two determinations were made in each sample and the mean taken.

Haemoglobin estimations: In order to make an accurate assessment of both the mean corpuscular haemoglobin concentration (M.C.H.C.) value and the degree of the anaemia, it was necessary to use a dependable, consistent and reproducible method to determine the haemoglobin concentrations.

In non-mammalian erythrocytes, the nuclei give rise to interference with several haemoglobin estimation procedures, notably with the popular acid haematin technique (Bell, Bird and McIndoe, 1965). In the present studies, a modification of the method described by Bell and colleagues was used. The method was found to be very simple, reproducible and reliable. It is a modification of the alkaline haematin method for total haemoglobin estimation, and neither the nuclear material of avian erythrocytes, nor the highly lipaemic plasma of the laying hen, causes interference.

O.1 ml./

0.1 ml. of well-mixed whole blood was pipetted into 0.9 ml. distilled water, and 34 ml. of 0.1 N sodium hydroxide solution added. After mixing, the mixture was incubated at 37°C in the water-bath for one hour then quickly cooled to room temperature. The absorption at 406mμ was then measured from an aliquot sample on an EEL Spectrophotometer. All readings were to be completed within 30 minutes on cooling to room temperature. Readings were then compared with a standard calibration curve, constructed from an isolated, purified chicken haemoglobin of the same strain of birds, and according to the method described by Allan (1969).

Preliminary trial estimations of haemoglobin concentrations of blood samples derived from normal chickens by this modified method compared well with published data, but this method had the added advantage of improved reproducibility of values.

Erythrocyte Counts: Preliminary total erythrocyte counts were made, using both the standard procedure with the blood diluted in formol-citrate solution (Dacie and Lewis, 1968), and the method described by Natt and Herrick (1952). Eventually, the latter method was preferred as it permitted the ready differentiation of the several types of the chicken erythrocytes, thus enabling direct counting of the erythrocytes and leucocytes from the same blood sample. Moreover, this method had specially been developed for avian blood cell counting.

Leucocyte Counts: The total leucocyte counts (and occasionally, the total thrombocyte counts) were made using the method described by/

by Natt and Herrick (1952) (vide supra).

The haemocytometer used in all these procedures was the improved Neubauer Counting chamber.

Reticulocyte Counts: For the estimation of the degree of reticulocytosis, the standard method described by Dacie and Lewis (1968), using 1 per cent brilliant cresyl blue for the supravital staining of the reticulocytes and other erythroblasts, was used.

Estimation of Absolute Haematological Values: These values were calculated using the formulae given by Archer (1965).

Post-mortem examination: Individual chickens were weighed before the start of these studies and upon the death of infected animal autopsy was immediately performed: the spleen and liver were removed, rinsed in physiological saline, dried between large Whatman filter papers, and finally weighed. At the same time, paired control chickens were killed and treated similarly. The spleen:liver ratio and the percentage of carcass weight of these organs were also determined.

Preparation of rabbit anti-chicken whole serum: Anti chicken whole serum was prepared in adult albino rabbits by a series of alternate daily, intravenous injections of normal chicken serum commencing at 0.1 ml. and gradually increasing the volume to 1 ml. Seven injections were given in all, and the rabbits were bled 5 to 7 days after the final injection.

The antiglobulin was standardized before use against erythrocytes sensitized in-vitro with S.gallinarum polysaccharide.

Detection of in-vivo erythrocyte sensitization: The indirect and direct Coombs' tests (ICT and DCT respectively) were employed in the detection of alterations ~~in~~ the erythrocyte surface during acute/

acute infection.

(a) Indirect Coombs' Test (ICT): Erythrocyte samples from infected and control chickens were washed 3 times in physiological normal saline (0.85 per cent) and titrated against serially diluted S.gallinarum antiserum. This antiserum, a pooled fraction from a group of chronic surviving chickens, was diluted before-hand to give a final haemagglutinating titre of 1:10240. The erythrocytes in those serum dilutions which showed no haemagglutination were then washed 3 times in large volumes of normal saline. To each of the tubes was added one drop of the standardized dilution (1:40) of the rabbit anti-chicken serum which had been previously absorbed with normal chicken erythrocytes to remove natural haemagglutinins. The tubes were shaken gently, incubated at 37°C for 30 minutes, and read.

(b) Direct Coombs' Test (DCT): Washed erythrocytes from infected and normal animals were titrated against serially diluted, adsorbed, heat-inactivated rabbit anti-chicken whole serum, using the single-drop method. The tubes were incubated as above, and the anti-globulin haemagglutination titres read.

(c) The Sensitivity of ICT and DCT: Before attempting to observe and detect in-vivo sensitization of erythrocytes in the infected chickens, preliminary experiments were carried out to observe the sensitivity of the tests to be employed. It was considered that the ICT and DCT would be useful tests for detecting small amounts of antigen on the surface of erythrocytes, as had been found with bacterial products (Buxton, 1959a; Young et al., 1962; Springer and Horton, 1964), penicillin (Ley, Harris, Brinkley, Liles, Jack and Cahan, 1958) and incomplete IgG, warm auto-antibodies (Dacie and Lewis, /

Lewis, 1968).

Standardization of ICT: Equal volumes of erythrocytes were sensitized against decreasing concentrations of alkali-treated S.gallinarum polysaccharide extract (Chapter III). The sensitized cells were then subjected to haemagglutination (HA) and anti-globulin HA tests (ICT) against S.gallinarum antiserum. The results of such an experiment are recorded in Table 1, which illustrates the greater sensitivity of the indirect antiglobulin HA test for detecting antigen adsorbed on erythrocytes. It also shows the effect of concentration of antigen employed in sensitization, demonstrating clearly that optimal concentrations must be employed for maximal sensitivity and specificity.

Standardization of DCT: Erythrocytes sensitized with the polysaccharide were subjected to haemagglutination test against S.gallinarum antiserum (Chapter III). The non-agglutinated cells were then thoroughly washed and subjected to direct haemagglutination against decreasing dilutions of the adsorbed rabbit anti-chicken whole serum. Typical results are shown in Table 2; clearly they demonstrate the sensitivity of antiglobulin serum to detect minute quantities of adsorbed globulins, probably the incomplete, non-haemagglutinating type.

(The optimal concentrations and conditions for the tests recorded were subsequently used in all further tests carried out throughout these studies.)

RESULTS

The results obtained from the chickens infected with S.gallinarum are summarised in Tables 3 and 4. They show the/

TABLE 1

Sensitivity of the Antiglobulin Haemagglutination Test (ICT) for Detecting Erythrocytes Sensitized with *S. gallinarum* Polysaccharide.

CONCS. OF LIPOPOLYSACCH.	DILUTIONS OF SALM. GALLINARUM ANTISERUM											CONTROLS
	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{500}$	$\frac{1}{10240}$	$\frac{1}{20480}$	$\frac{1}{40960}$	$\frac{1}{81920}$	$\frac{1}{16384}$	$\frac{1}{327680}$	$\frac{1}{645360}$	
1000	4	4	4	3	2	+++	+++	+++	+++	+++	++	-
100	4	4	3	1	+++	+++	+++	+++	++	+	-	-
50	4	4	2	+++	+++	+++	++	++	+	-	-	-
25	2	+++	+++	++	++	+	-	-	-	-	-	-
10	+++	+++	++	+	±	-	-	-	-	-	-	-
5.	++	+	-	-	-	-	-	-	-	-	-	-
2.5	+	-	-	-	-	-	-	-	-	-	-	-
1.0	-	-	-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-	-	-	-	-
HAEMAGGLUTINATION (HA)		ANTIGLOBULIN HAEMAGGLUTINATION						SALINE ANTIGLOBULIN CONTROLS				
4 = STRONG HA.		+++ = STRONG ANTIGLOB. HA.						(i) MIXTURES OF SENSITIZED CELLS AND ANTIGLOBULIN SERUM				
3 = MODERATE HA.		++ = MODERATE ANTIGLOB. HA.						(ii) MIXTURES OF NON-SENSITIZED CELLS AND SALM. GALLINARUM ANTISERUM				
2 = WEAK (SLIGHT) HA.		+ = WEAK (SLIGHT) ANTIGLOB. HA.						(iii) MIXTURES OF NON-SENSITIZED CELLS AND ANTIGLOBULIN SERUM				
1 = TRACE HA.		± = TRACE ANTIGLOB. HA.										

TABLE 2

Sensitivity of the Anti-globulin Serum for Detecting
Erythrocytes Sensitized with Globulins.

DILUTIONS OF ANTI-GLOBULIN	DILUTIONS OF SALM. GALLINARUM ANTISERUM									
	$\frac{1}{2560}$	$\frac{1}{500}$	$\frac{1}{10240}$	$\frac{1}{20480}$	$\frac{1}{40960}$	$\frac{1}{81920}$	$\frac{1}{16384}$	$\frac{1}{327680}$	$\frac{1}{645360}$	
1/5	4	4	2	+++	+++	+++	++	++	-	
1/10	4	4	2	+++	+++	+++	++	++	+	
1/20	4	3	2	+++	+++	+++	++	+	±	
1/40	4	4	2	+++	+++	+++	+	+	-	
1/50	4	4	2	+++	+++	+++	+	+	-	
1/60	4	4	2	+++	+++	+++	+	+	-	
1/70	4	3	2	+++	+++	+++	+	+	-	
1/80	4	4	2	+++	+++	+	+	-	-	
1/100	4	3	2	+++	++	+	+	-	-	

HAEMAGGLUTINATION (HA)	ANTI-GLOBULIN HAEMAGGLUTINATION
4 = STRONG HA.	+++ = STRONG ANTIGLOBULIN HA.
3 = MODERATE HA.	++ = MODERATE ANTIGLOBULIN HA.
2 = WEAK (SLIGHT) HA.	+ = WEAK (SLIGHT) ANTIGLOBULIN HA.
1 = TRACE HA.	± = TRACE ANTIGLOBULIN HA.

the extreme virulence of the bacterial strain used; all the animals infected died within 6 days, and 50 per cent of the total number of deaths occurred on the fifth day (Fig. 1).

Because of the great variations in the published data on the normal haemograms of chickens (Diesem Venzke and Moore, 1958; Lucas and Jamroz, 1961) it was decided that the normal values obtained for the studies reported here would be the basis for comparison of any deviation from the normal as a result of the infection. Indeed, it has been suggested recently that such a procedure is probably the main criterion for objective assessment of the abnormal (Osbaldiston, 1968). Therefore, preliminary studies involving numerous sampling of healthy chickens using the various techniques described above to obtain normal data, were made before the animals were finally infected.

(1) Clinical Manifestations:

The present experiments showed all the characteristic symptoms normally associated with this disease.

The first noticeable symptom was the appearance of a sulphur-yellow colouration of the droppings, which was very slight at first. Later, as the consistency of the droppings became watery, the alteration in colour was a most marked yellow, with an additional greenish tinge. There was little or no variation in the onset and severity of the diarrhoea. There was also inappetance, extreme dejection, and weakness. They appeared listless and motionless for long periods with feathers ruffled and head sunk on the chest, and both eyes were closed. The temperature in some chickens was very high, reaching 115°F at the peak of infection, often falling to subnormal levels just before death.

Severe/

RELATIONSHIP BETWEEN DURATION of FATAL INFECTION and
TYPE of R.B.C. SENSITIZATION

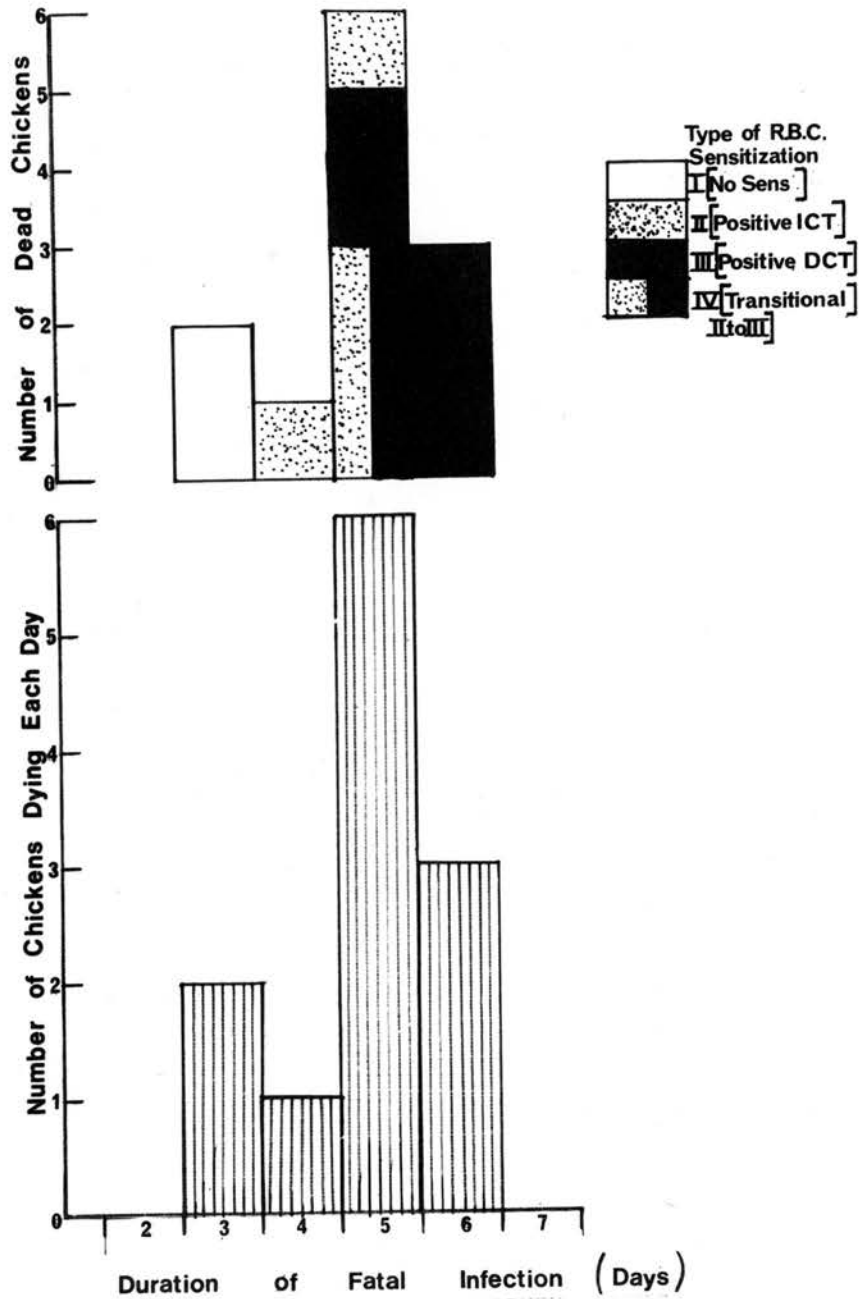


FIGURE 1. The Relationship between the Type of in-vivo Sensitization of Erythrocytes and the Time of Death after Infection.

Severe pallor of the comb and wattles, followed by cyanosis, was a common symptom. There was respiratory difficulty with distinct dyspnoea. Collapse, with convulsive movements of the limbs, and defaecation was rapidly followed by death.

(2) The Mortality Rate:

The survival times of chickens fatally infected are illustrated in Fig. 1. All the animals became severely ill before death. Deaths were first observed on the third day after infection, and the death rate then increased rapidly, reaching a peak on the fifth day, when 50 per cent of the total number of infected animals died. The rest of chickens died the following day. Thus, within 7 days all the infected chickens had died.

(3) Pathological Changes in the Blood following Infection:

Every animal examined showed the classical triad of anaemia, reticulocytosis and hepato-splenomegaly. This was substantiated by autopsy findings of haemosiderosis of the spleen and liver.

From Table 3 it will be observed that the anaemia was severe in nearly all the chickens. As this was accompanied in all cases by reticulocytosis and enlargement of the spleen and liver, this suggested that it was of the haemolytic variety and not dyshaemopoietic.

It was also considered highly significant that the anaemia coincided with onset of severe symptoms and death (Table 3 and Fig. 1). In addition, clinical signs of anoxia, anaemia and extreme cyanosis suggest that a pathological abnormality of the blood may be intimately associated with the pathogenesis of the disease syndrome.

(a)/

(a) Changes in the Haematocrit: During infection, there was nearly 50 per cent fall in the haematocrit levels (36 ± 1.3 to 18.3 ± 3.3 per cent) as shown in Table 4. The fall in PCV was particularly severe in chicken number 42, which initially had a PCV of 35, but a few hours before death dropped dramatically to 11 per cent (Table 3). This chicken died on the fifth day after infection, and death, like the other infected animals, coincided with the sudden fall in the haematocrit level (Fig.2)

(b) Changes in Haemoglobin Concentration: As with haematocrit, haemoglobin levels dropped suddenly and dramatically just before death (Fig.3). On the average, haemoglobin concentration of 12.6 ± 0.78 dropped to a final value of 7.0 ± 1.4 gms. per 100 ml. of blood (Table 4). A number of preliminary trials were made from healthy normal chickens to obtain same data on normal haemoglobin levels. It was found that haemoglobin concentration for hens between 2 and 4 months varied greatly between 11 and 12 gms. per cent. On the other hand, relatively higher values were obtained for hens more than 5 months old (between 12 and 14 gms. per cent). The latter figures compared well with estimations of haemoglobin levels in chickens by Buxton (1960).

The data on haemoglobin estimations during infection are summarized in Tables 3 and 4, and also illustrated in Fig. 3. They show that there was little or no variation from the normal until clinical signs developed shortly before death. There was, however, a massive attempt to compensate for this severe loss as indicated by the sharp rise in haemoglobin concentration during recovery. This apparent increase in haemoglobin concentration was found to be due largely to the reticulocytosis which occurred at this/

TABLE 4

TABLE OF FINAL ACUTE INFECTION PARAMETERS AT TIME OF DEATH

ACUTE INFECTION PARAMETERS			INFECTED (N = 12)			CONTROLS (N = 12)		
			MEAN	SD	RANGE	MEAN	SD	RANGE
A	HAEMOGLOBIN CONC. (gms/100ml BLOOD)		7.0	1.40	4.4 - 9.2	11.24	0.33	10.9 - 11.8
B	HAEMATOCRIT (P.C.V.) (%)		18.30	3.34	11.0 - 25.5	33.60	1.0	32 - 35
C	TOTAL ERYTHROCYTE COUNT ($\times 10^6/\text{mm}^3$)		0.97	0.35	0.4 - 1.8	2.60	0.10	2.4 - 2.7
D	RETICULOCYTE COUNT (%)		36.60	6.0	23.5 - 45.1	10.60	1.84	6.9 - 13.1
E	TOTAL LEUC ^{LEUC} OCYTE COUNT ($\times 10^3/\text{mm}^3$)		189.70	59.70	88 - 266	36.0	2.41	34 - 42
F	ABSOLUTE HAEMATOCRIT ^{LEUC} VALUE	(i) M.C.V. (μ^3)	201.50	41.80	141.7 - 283.3	131.84	5.07	125.0 - 140
		(ii) M.C.H. (μ^2)	77.50	15.50	56.7 - 110	44.13	1.80	41.9 - 47.2
		(iii) M.C.H.C. (%)	38.53	1.24	35.9 - 40.0	33.50	0.58	32.8 - 34.7
G	SPLEEN/LIVER RATIO:		0.106	0.016	0.075 - 0.13	0.042	0.0043	0.034 - 0.050
H	% OF CARCASE WEIGHT	(i) SPLEEN	0.532	0.074	0.34 - 0.62	0.176	0.022	0.15 - 0.2
		(ii) LIVER	5.44	0.50	4.4 - 6.4	2.40	0.186	2.1 - 2.7

this stage (Fig.4).

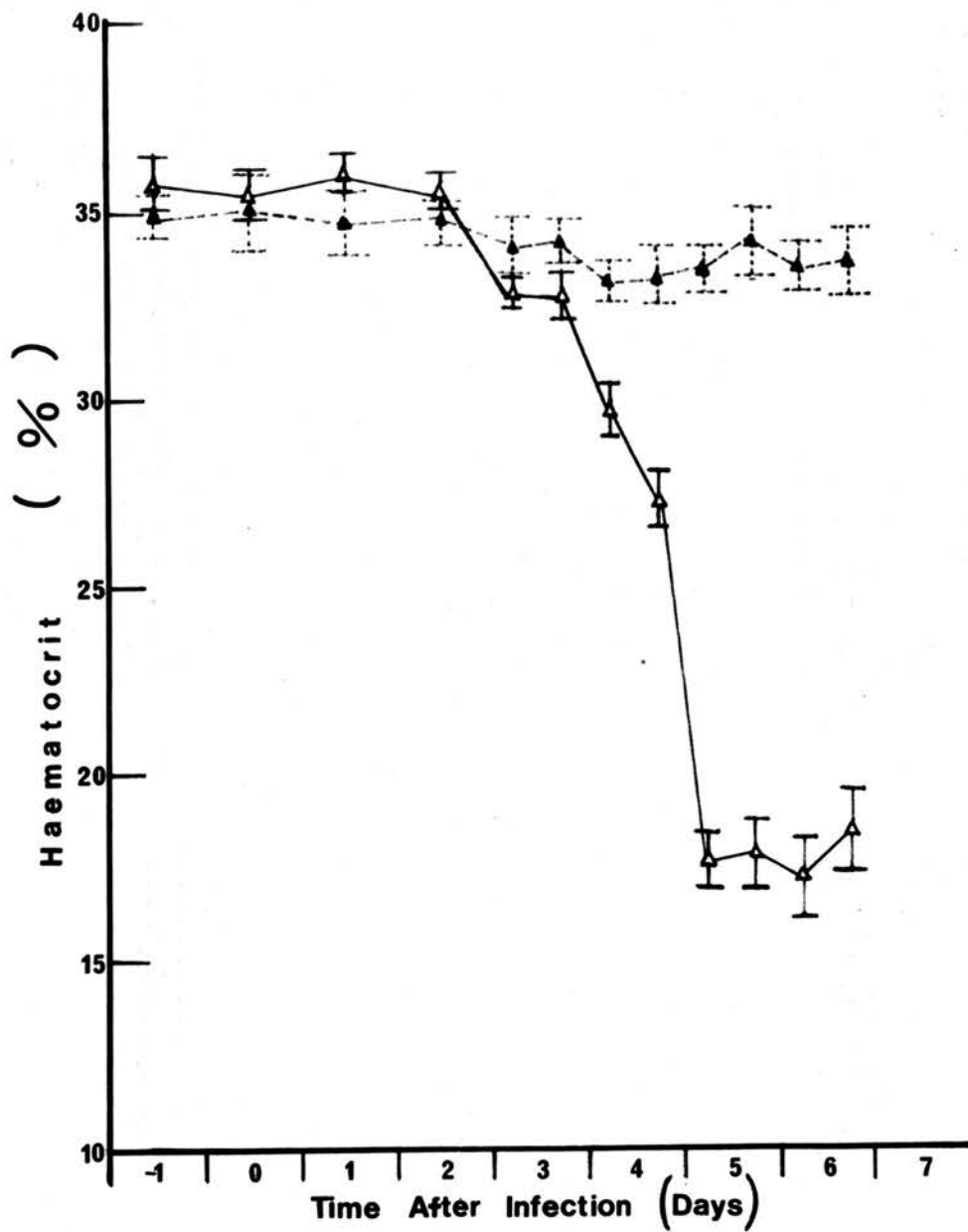
(c) Changes in Erythrocyte Volume: Fig.5 indicates the sudden loss of erythrocytes at the peak of infection. The total loss was very substantial — from an average of 2.8 ± 0.09 to 0.97 ± 0.35 million cells per cu.mm. at time of death (Table 4).

(d) Reticulocytosis: The discrepancies in the published data of the normal blood levels of avian reticulocytes may largely be due to the use of "reticulation" — the number of reticular materials or 'dots' — as a basis for identification. Thus Coutes and Marsh (1966) give the mean count as between 8 and 15 per cent on the basis of identifying the cell as 'reticulocyte' if the cell has a single 'dot'.

In the present study, counts given represent the number of cells showing "reticulation" of more than 5 to 6 dots. This designation, even though it increased the subjectivity of reticulocyte counting, nevertheless limited the serious error of identifying all these cells as reticulocytes regardless of the number of dots present.

Unusually high reticulocytosis was a common finding in this disease (Fig.6). A six-fold increase in the total circulating reticulocytes was estimated (Tables 3 and 4), and the peak of erythropoietic activity coincided with the onset of the clinical symptoms of a severe anaemia.

Histological examination of the bone-marrow, spleen and liver in the course of these studies revealed gross hyperplasia of the marrow and extra-medullary erythropoiesis in the spleen and liver. This increased erythropoietic activity — reflected in reticulocytosis — indicates that the observed clinical anaemia is/



* Bars through each point represent the Standard Error (S.E.) of the Mean.

FIGURE 2. The Changes in Haematocrit During Acute Fowl Typhoid.

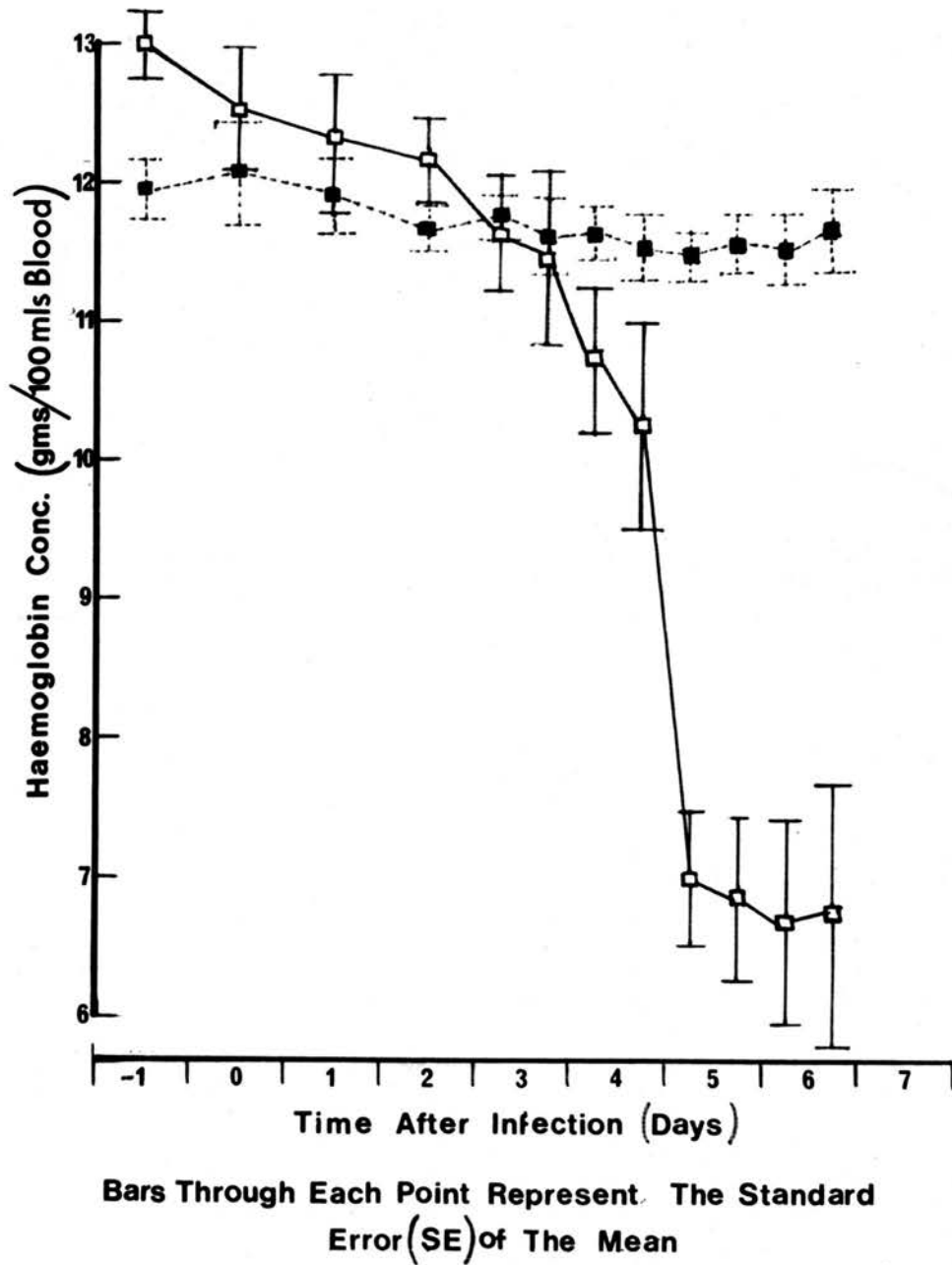


FIGURE 3. The Changes in Haemoglobin Concentration During Acute Fowl Typhoid.

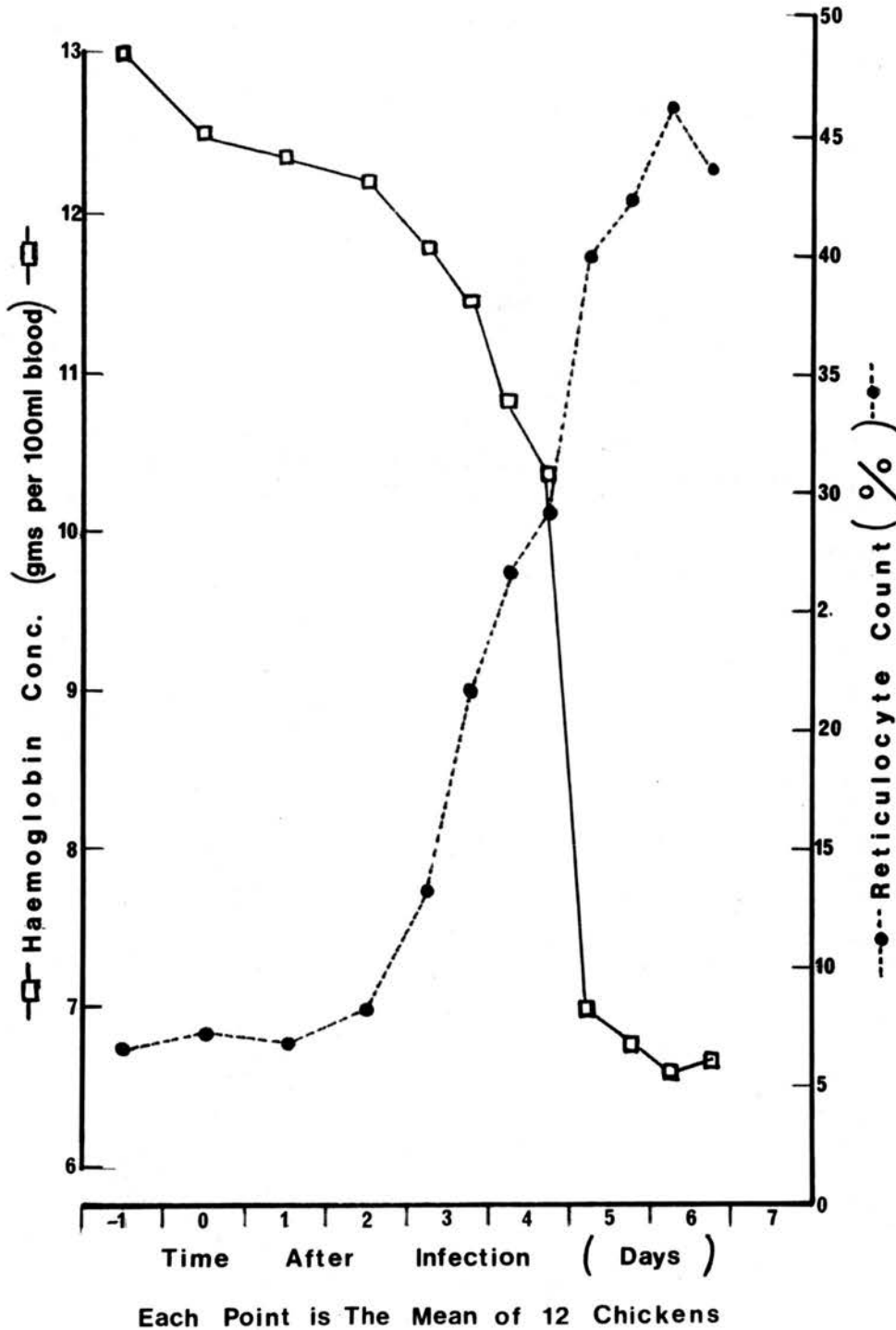
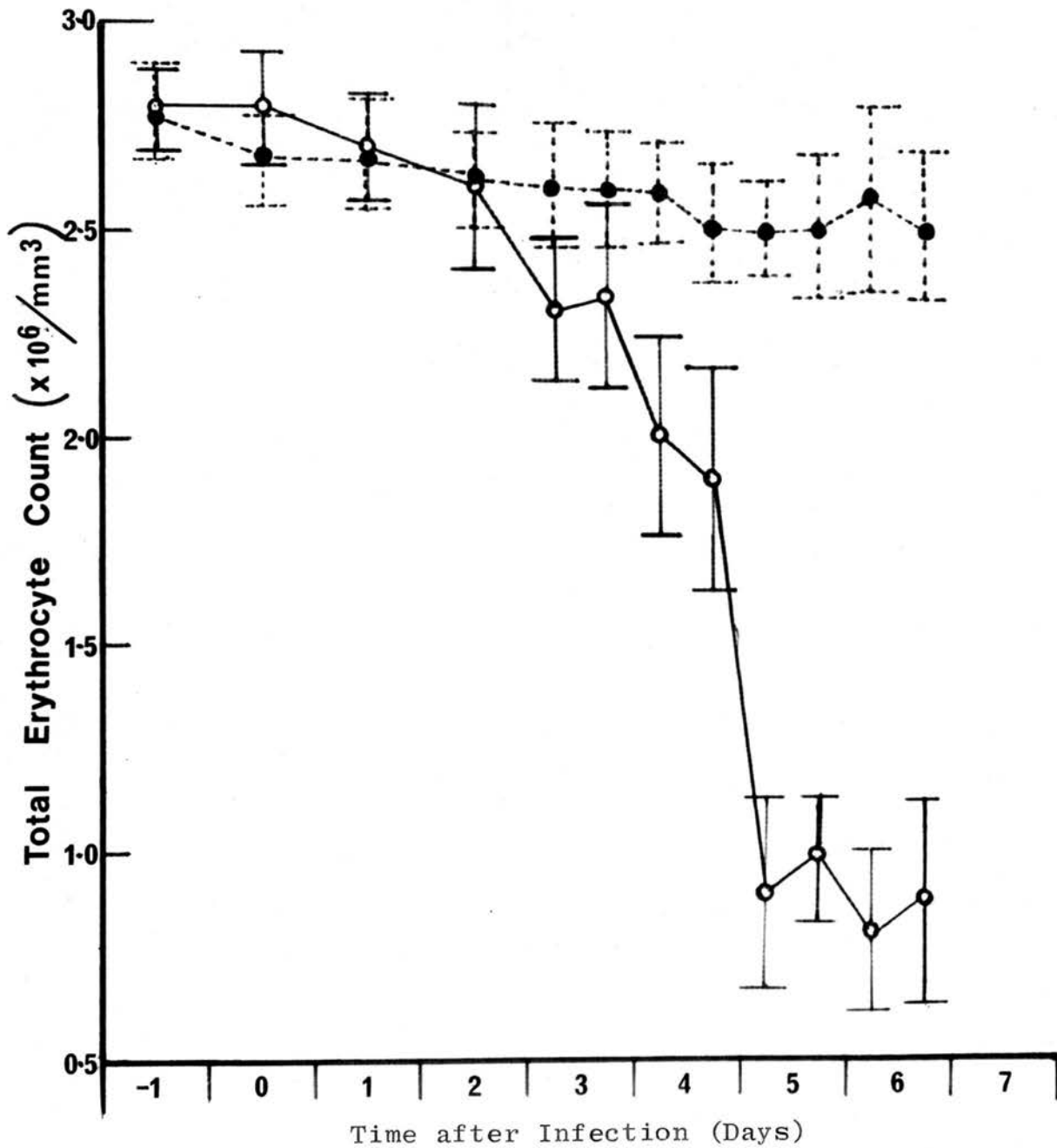
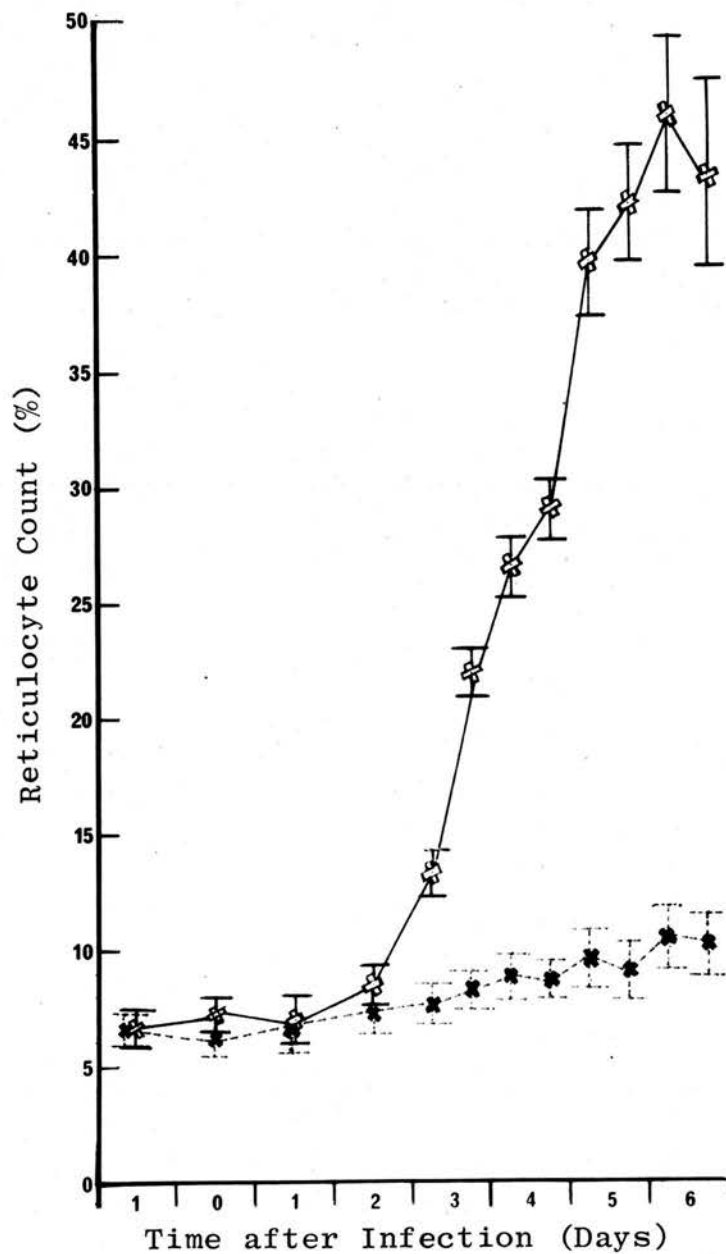


FIGURE 4. The Relationship between the Activity of the Haemolytic Process (Haemoglobin Conc.) and the Degree of Attempted Repair (Reticulocytosis).



* Bars through each point represent the Standard Error (S.E.) of the Mean.

FIGURE 5. The Variations in Total Erythrocyte Count During Acute Fowl Typhoid.



*Bars through each point represent the Standard Error (S.E.) of the Mean.

FIGURE 6. The Degree of Reticulocyte Response During Acute Fowl Typhoid.

is not due to inhibition of marrow activity and therefore that it is not of the dyshaemopoietic type.

Fig.4 demonstrates the attempts of the host to compensate for the increased destruction of erythrocytes by premature entry of reticulocytes and other immature cells into the general circulation.

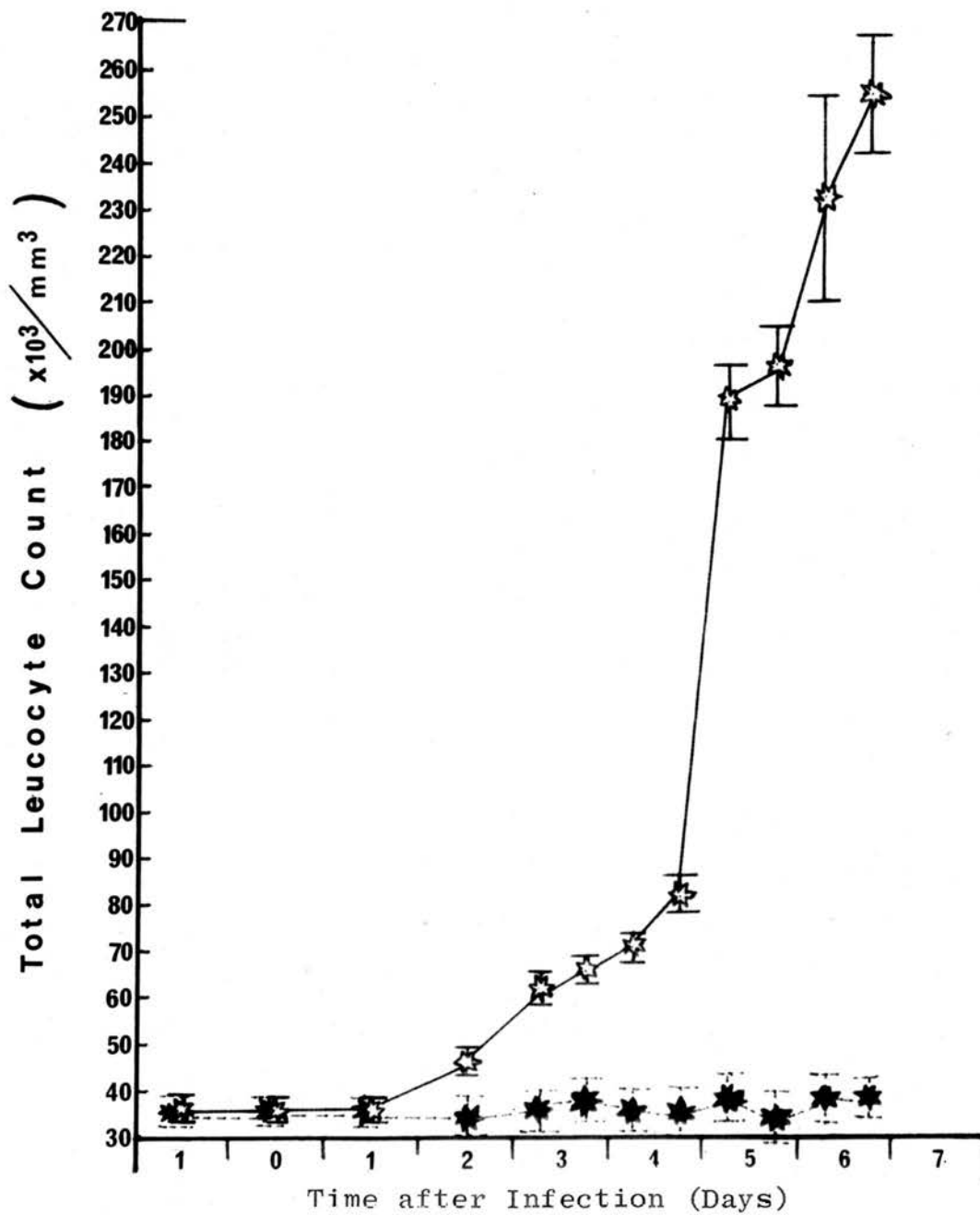
(e) Alterations in Blood Leucocyte Levels: During the acute disease significant changes also occur in the leucocyte levels (Fig.7). Massive leucocytosis, essentially neutrophilia, was estimated as nearly a five-fold increase (357 ± 1.9 to $189.7 \pm 59.7 \times 10^3$).

The lymphocyte level was decreased and also occasionally, the thrombocyte count was found to be well below the normal value.

(f) Applications of the Absolute Values: Although the value of these "ratios", especially the MCV, in diagnosis and classification of the anaemias depends largely on the accuracy of the counting of the total erythrocytes, it was nevertheless considered useful to have an idea of any changes in both the erythrocyte volume and haemoglobin concentration.

The mean corpuscular volume (MCV) was nearly doubled (131.84 ± 5.07 to $201.5 \pm 41.8 \mu^3$) and a similar increase also occurred in the mean corpuscular haemoglobin (MCH) — from 44.13 ± 1.8 to $77.5 \pm 15.5 \mu^2_{gm}$. (Table 4). In contrast, the mean cell haemoglobin concentration (MCHC) was unchanged — 33.5 ± 0.58 to 38.53 ± 1.24 per cent. These estimations indicated that the anaemia is macrocytic but normochromic, with some evidence of polychromasia.

(g) The Detection of in-vivo Sensitized Erythrocytes: Routine examination of the erythrocytes for sensitization during infection revealed/



* Bars through each point represent the Standard Error (S.E.) of the Mean.

FIGURE 7. The Changes in Leucocyte Count During Acute Fowl Typhoid.

revealed that at the peak of infection, and a few hours before death, the erythrocytes became sensitized, as was shown by the demonstration of either positive ICT or DCT. The distribution of the different types of sensitization during infection, and their relationships with the acuteness of the disease is shown in Fig.1. The figure suggests a possible relationship between the type of sensitization of erythrocytes, the time of death and probably also, the severity of the anaemia. For instance, the fifth day after infection, when the majority of chickens showed sensitization, was also the peak of mortality since 50 per cent of the total infected animals died at this time (Fig.1). It was noteworthy that those chickens which died within 3 days (for example number 30H and 33H)(Table 3) showed no erythrocyte sensitization, and the anaemia was less severe. On the other hand, those chickens dying later than 3 days had their erythrocytes sensitized in-vivo, and the anaemia associated with this group was extremely severe. The period after infection, during which the cells became sensitized in-vivo (fourth to the sixth day) (Fig.1), coincided with the period of greatest destruction of erythrocytes and hence, the appearance of the peak of anaemia (Tables 3 and 4).

(4) Pathological Changes in other Organs:

Gross enlargement of the spleen and liver was one of the commonest autopsy findings (Fig.8). Contrary to the observation of Wilson (1946), bronzing of the liver was found to be a common and immediate post-mortem finding (Fig.9). Occasionally, the colouration was so intense as to impart a greenish appearance to both the plasma and the liver as a result of biliverdinaemia (Wintrobe, 1967). Generally, the spleen size had trebled and liver/

SPLENO-HEPATOMEGALY

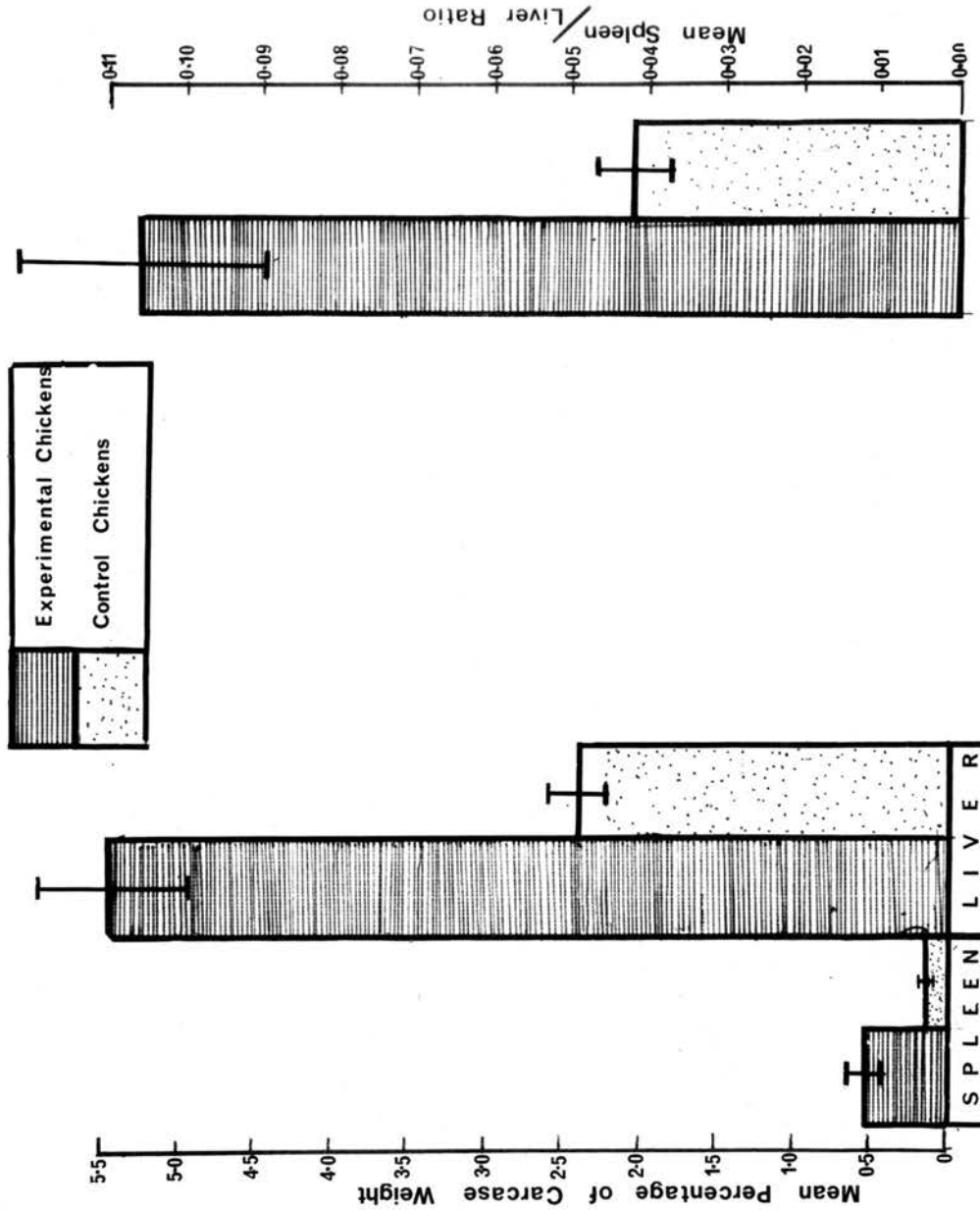


FIGURE 8. Hepato-splenomegaly in Fowl Typhoid. Gross enlargement of the spleen (3 x) and the Liver (2 x). Also shows general increase in $\frac{\text{spleen}}{\text{liver}}$ ratio.



FIGURE 9. Gross Appearance of Spleen and Liver Following Infection with *S. gallinarum* (Chicken Nos. 37 - 41) compared with Control Chickens (Nos. 37C - 41C). Note typical 'bronzing' (sometimes greenish) colouration of the liver. Taken 24 hours after autopsy, hence 'discoloured' control spleen.

liver weight nearly doubled (Fig. 8) at the time of death. Tables 3 and 4 also demonstrate the increases in the spleen/liver ratio.

Other pathological changes were the marked inflammatory changes in the intestines. In per-acute cases there was a haemorrhagic duodenitis, whereas a reddening and swelling of the mucous membranes, sometimes extending throughout the entire length of the small intestines were associated with the acute cases. The small intestines in the latter cases were occasionally accompanied by a shiny, reddish-green gelatinous exudate.

DISCUSSION

The results of these experiments have demonstrated the extreme susceptibility of adult chickens to S.gallinarum infection. The intramuscular route of infection was found to be the most suitable for these and subsequent experiments in that it ensured both the reproducibility of high mortality and ease of initiation of acute infection. Some previously reported data on this subject (Wilson, 1946; Gordeuk, Glantz and Callenbach and Thorp, 1949; Smith, 1955; Buxton, 1959^b) has shown that an oral challenge of chickens with S.gallinarum would produce either a mild or severe disease. In contrast, these studies show that a very severe and acute infection is almost always produced if the animals were challenged intramuscularly. Peak mortality was found to be between the fourth and sixth days after infection (Fig.1).

The present results also confirmed and extend the characteristic/



characteristic clinical and pathological signs reported by other workers. Thus, Wilson (1946) had noticed greenish-tinged, sulphur-yellow colouration of the droppings, cyanosis of comb and wattles, enteritis and typical 'bronzing' of the liver. Similar observations were made by Smith (1955), who also found that the gall-bladder and the bile never showed any infection. In this respect, this disease differs from many of the salmonella infections in man (Wilson and Miles, 1964) and animals (Buxton, 1957).

Buxton (1960) has also reported that, shortly before death, the symptoms always observed were those of extreme thirst, loss of weight, inappetance, diarrhoea, cyanosis of comb and wattles, laboured respiration, and sudden collapse. This author also suggested the possibility that these clinical signs might be due to the direct result of the toxic products from bacterial multiplication and disintegration in the tissues. It is noteworthy that nearly identical symptoms have been observed in rabbits after intravenous injection of lipo-polysaccharide (Landy, Skarnes, Rosen, Trapani and Shear, 1957). It is therefore tempting to conclude that the symptoms and death of the chickens are consequent upon the ability of the bacteria to multiply and to disintegrate rapidly in the host.

Observations in the present studies also indicate that the anaemia is a most important, if not the most important pathological symptom in this disease. The typically anoxic signs of cyanosis, laboured respiration and sudden collapse undoubtedly contributed to the cause of death. It is therefore conceivable that many of the observed clinical signs in this disease may be directly due to the effects of the severe anaemia. Interestingly, these/

these signs were not unlike the signs observed by Jandl (1957) and Jandl and Tomlinson (1958) when erythrocytes were destroyed by antibodies in man. They showed that fever, leucopaenia, dyspnoea, dermal reactions, renal injury, gastro-intestinal disturbances and release of histamine and serotonin were characteristic of the normal host response to immune haemolysis as these signs were analogous in their pathogenesis to the host responses to other antigen-antibody reactions and to bacterial lipo-polysaccharides. Significantly, these authors also found that haemolysis of comparable degree by non-immunologic mechanisms did not cause these signs.

The anaemia was very severe, but Fig.4 shows the attempt by the host to compensate and repair the damaged erythrocytes by the release into the circulation of immature blood cells, particularly reticulocytes. Reticulocytosis was a reflection of increased erythropoietic activity of the bone-marrow and other extra-medullary erythropoietic organs, especially the spleen and liver. These observations demonstrate clearly that the anaemia associated with acute fowl typhoid is definitely not due to inhibition of erythropoietic activity.

Evidence from the erythrocyte absolute value determinations had shown that the anaemia is both macrocytic and normochromic but this anaemia has never fully previously been characterised. In 1934, Cook and Dearstyne in a study of the pathological changes occurring in the blood during acute fowl typhoid, reported a severe anaemia, gross heterophilia and lymphopaenia. No evidence was, however, presented to demonstrate the severity or characterize the type of anaemia. Earlier however, Ward/

Ward and Gallagher (1920) had observed nearly 50 per cent reduction in the total circulating erythrocyte numbers during this disease. They also made the interesting finding that numerous leucocytes were seen "to be attacking the erythrocytes". This obviously indicated a possible erythrophagocytosis by the circulating leucocytes. Studies by Zinkham and Diamond (1952), Conway (1953) and Jandl and Tomlinson (1958) have shown that this type of in-vivo erythrophagocytosis is common in many haematologic diseases. Observations made during the course of the present studies, but not recorded here, demonstrated that erythrophagocytosis occurred in the blood stream at the peak of infection and anaemia. When, at the time of detection of in-vivo sensitization, blood samples were taken from the infected animal and stained, either incubated or not, leucocytes, mainly the mononuclear type, were found to be engulfing erythrocytes. Whether this phenomenon contributes significantly to the production of the anaemia is conjectural, but there are interesting reports in the literature (Zinkham and Diamond, 1952; Conway, 1953; Jandl and Tomlinson, 1958) which indicate that this type of erythrophagocytosis may be more important in certain haemolytic syndromes than hitherto imagined. In general, however, it seems likely that the circulating leucocytes may aid the fixed macrophages in the reticulo-endothelial system (RES) in destroying trapped, sensitized erythrocytes (Swisher, 1964; Leddy, 1966; Rifkind, 1966).

It was found during the present studies that in-vivo erythrocyte sensitization was detectable only at the peak of anaemia (Tables 3, 4 and Fig.1). This suggests a causal relationship between sensitization of erythrocytes and the anaemia. Buxton (1959a)/

(1959a) had recognised this possibility, and in a later study (Buxton 1960) suggested that the anaemia associated with acute fowl typhoid is a direct result of the increasing ability of the RES to take up liberated bacterial polysaccharide, as well as sensitized erythrocytes. On the other hand, the anaemia might be initiated by modification to the erythrocyte surface other than to RES hyperfunction per se. The contribution to the production of the anaemia by the increase in the RES activity would therefore be secondary to the primary event of in-vivo sensitization of the erythrocytes. Evidence that this, in fact, may be so is presented later (Chapters V and VII).

However, it was of interest also to investigate the significance, and the probable role of the gross splenic and hepatic enlargement in the pathogenesis of this anaemia. The question arises as to whether this enlargement is the result or the cause of the increased sequestration and destruction of the sensitized erythrocytes within these organs. In this connection, the studies of Jandl, Jacob and Daland (1961) are pertinent. These authors, in a study of various infections in which varying erythrocyte survivals and increased splenic sequestration were demonstrated, suggest that the accompanying splenomegaly was the cause of the observed increased haemolysis. Similar conclusions and suggestions have also been made by Sherman and Friedell (1962) and Wagner, Iio and Hornick (1963) and recently, Jacob (1966) has argued in favour of the thesis that splenomegaly, regardless of cause, probably results in decreased erythrocyte survival. Buxton (1960) has invoked a similar hypothesis to explain the causation of the anaemia in acute fowl typhoid. Furthermore, Ho and Kass (1958)/

(1958) have suggested that hypersequestration by the non-specifically activated RES probably accounted for the haemolytic anaemia which developed in rabbits after intravenous injection of endotoxin.

Satisfactory direct evidence has, however, been very difficult to obtain in favour of this attractive hypothesis. It may well be that splenic enlargement is the primary event in some of these reported cases and that the increased haemolysis was secondary to it. Nevertheless, it is also quite possible that the reverse may be the true explanation. Indeed, subsequent observations in this thesis (Chapters V and VII) indicate that hepato-splenomegaly plays a passive rather than an active role, in the production of the anaemia in this particular disease. Thus, the effect of any stimulation of phagocytic activity of the RES, if this occurs during this infection, may therefore merely be to enhance the rate of sequestration of the in-vivo sensitized cells. The mechanisms operating to destroy these erythrocytes ~~in~~ extra-vascular, as evidenced by the absence of obvious intravascular haemolysis and presence of biliverdinaemia and "bronzing" of the liver.

An interesting association which emerged from the studies presented here, was the apparent relationship between the type of erythrocyte sensitization (Chapter III), the rate of mortality and the severity of the anaemia (Fig.1). It was found that erythrocyte sensitization detectable by ICT appeared first, and subsequently with sensitization detectable by DCT, and this coincided with the peak of severity of the clinical signs, including the anaemia. Chickens in which a change from positive ICT to positive/

positive DCT was observed, often appeared to have less severe symptoms and occasionally recovered. Evidence was, however, insufficient to conclusively establish a meaningful pattern of relationships, and further work was undertaken to confirm and extend these observations. The results of this study form part of the results presented in the next chapter.



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CHAPTER IIISIGNIFICANCE OF IN-VIVO ERYTHROCYTE SENSITIZATION IN THEDEVELOPMENT OF THE HAEMOLYTIC ANAEMIAINTRODUCTION

The previous chapter confirmed previous observations that in-vivo sensitization of erythrocytes occurs during acute fowl typhoid (Buxton, 1959a). The results also indicated that the severe anaemia which develops characteristically in the acute form of this disease may be due to increased destruction of altered erythrocytes rather than inhibition of erythropoietic activity. This conclusion follows from the findings that in-vivo sensitization of erythrocytes occurred coincidentally with the appearance of a severe anaemia, which suggested a causal relationship between this phenomenon and anaemia, and gross reticulocytosis and bone marrow hyperplasia were also always observed.

Although significant anaemia is frequently associated with chronic bacterial diseases, it is a less common event in the course of acute infections (Cartwright and Wintrobe, 1952; Weinstein and Beutler, 1962; Mitus, 1966; Mengel, Metz and Yancey, 1967; Editorial, 1967), and the mechanism is still largely undetermined. This type of anaemia was, however, once generally believed to be of the dyshaemopoietic type (Vaughan and Saifi, 1939; Saifi and Vaughan, 1944; Cartwright, Lauritsen, Jones, Merrill and Wintrobe, 1946; Wintrobe and Cartwright, 1952), the suggestion being that the disturbance of erythropoiesis was merely part of the general toxæmia or/

or disturbance of metabolism. However, Brown, Hayward, Powell and Witts (1944) and Brown (1950) have put forward evidence to show that the anaemia which complicates acute bacterial infections may be due to unduly excessive destruction of erythrocytes and have suggested that haemolysis was important in the pathogenesis of this type of anaemia. However, they did not report any accompanying abnormalities of erythrocytes and neither did they present any direct evidence of haemolysis.

Several significant observations of the occurrence of haemolytic episodes associated with positive direct Coombs' test (DCT) in enterobacterial infections, mostly in man, have also been reported (Berman, Braun and Rachmilewitz, 1945; Shaw, 1951; McFadzean and Choa, 1953; Horowitz, Javid and Spaet, 1960; Ruggieri, 1961; Retief and Hofmeyr, 1965; Dacie, 1967). There have also been few reports on observations of in-vivo sensitization of erythrocytes with no concomittant anaemia during the infection (Saito et al., 1961; Young et al., 1962; Springer and Ansell, 1962; Springer and Horton, 1964; Springer, 1964). The relationship of these observations to the mechanism of anaemia of gram-negative bacterial infections in general has only been inferred but never established.

The hypothesis is offered here that the in-vivo combination of sensitized erythrocytes with the induced antibody accelerated their rate of destruction by the RES and this may be responsible for the haemolytic episodes associated with the above bacterial infections. In particular, the phenomenon of opsonin-promoted erythrophagocytosis of in-vivo sensitized erythrocytes may be the main mechanism responsible for the haemolytic anaemia associated/

associated with acute fowl typhoid.

The present equivocal state of the knowledge of both the nature of the sensitizing factor(s) and the relationship of the sensitized erythrocytes to the pathogenesis of the anaemia prompted the following studies which had three main objectives:

- (1) To investigate further the relationship of in-vivo sensitization of erythrocytes in the development of the haemolytic anaemia.
- (2) To relate any abnormalities of erythrocytes to the immunological response to infection. It was considered that such correlative studies would throw some light on the possible immunological basis, if any, on the mechanism of anaemia.
- (3) To obtain further evidence which may confirm the demonstration in the previous chapter that there are different types of in-vivo erythrocyte sensitization and also to investigate the possibility that a relationship exists between the type of erythrocyte sensitization, the severity of the ensuing anaemia, and the duration of the infection generally (Chapter II). It was also intended to examine the possible significance of such relationships in the overall pathogenesis of the disease.

MATERIALS AND METHODS

Sampling, serological and haematological tests were as described in previous chapters.

Samples were taken once daily for the determination of the parameters, except in the detection of in-vivo sensitized erythrocytes when two or more samples were taken daily to determine the/

the 'type' of erythrocyte sensitization.

Erythrocyte Osmotic Fragility tests: The technique employed was essentially the same as that described by Buxton (1960), which was a modification of Dacie and Lewis' method (Dacie and Lewis, 1968). Stock solutions of buffered NaCl were obtained by dissolving NaCl (180 gms.) Na_2HPO_4 (27.31 gms.) and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (4.86 gms.) in 2 litres of triple-distilled water. The pH was adjusted to 7.4 and 1.0 per cent solution in distilled water was prepared from this stock solution, which was stored on ice. The following percentage dilutions were then made: 0.85 ("Blank"); 0.75; 0.60; 0.55; 0.50; 0.45; 0.40; 0.35; 0.30; 0.25; 0.20; 0.10; 0 ("100 per cent haemolysis").

Well mixed, aerated, heparinised whole blood was added in volumes of 0.05 ml. by means of a capillary pipette, to tubes containing 5 ml. volumes of each dilution of salt solution and also to 5 mls. of distilled water (for the "100 per cent haemolysis"). The mixtures were shaken, stored at room temperature (20°C) for 45 minutes and re-shaken at intervals during this time. After centrifugation at 2000 r.p.m. for 10 minutes the supernatant from each sample was examined in an EEL colorimeter. Recordings were taken using 10 mm. diameter tubes, a green filter (OGR) and a "Blank" obtained from the supernatant containing 0.85 per cent NaCl.

The degree of haemolysis in each tube was then compared with that in the 100 per cent lysis tube, and finally concentrations of NaCl were plotted against percentage incremental haemolysis as advocated by Suess, Limentani, Dameshek and Dolloff (1948) and Bolton (1949).

Indirect Haemagglutination (HA) and Antiglobulin HA Tests: These tests/

tests were used for the detection of specific serum antibodies during infection, and were also adapted to observe and estimate the degree of in-vivo erythrocyte sensitization.

(a) S.gallinarum Lipo-polysaccharide: Bacterial lipo-polysaccharide (O-antigen/or LPS) for sensitizing chicken erythrocytes and standardisation of the tests, was extracted from washed suspensions of the stock culture of S.gallinarum grown on nutrient agar in Roux flasks by the phenol-water procedure of Westphal, using the modified method described by Ravin, Rowley, Jenkin and Fine (1960).

(b) Sensitization of Erythrocytes: The washed erythrocytes were sensitized by incubating a 10 ml. 1.5 per cent suspension of cells at 37°C for one hour in 0.85 per cent saline containing 0.2 ml. of a 5000 µgm. per ml. alkali-treated S.gallinarum LPS. Alkali treatment improved erythrocyte sensitization (Neter, Westphal, Lüderitz and Gorzynski, 1956; Davies, Crumpton, Macpherson and Hutchinson, 1958), and this was carried out by dissolving a weighed quantity in 0.02N NaOH and heating to 37°C for 2 hours, after which the solution was re-neutralized with 0.1N HCl. After sensitization the erythrocytes were again washed 3 times in 0.85 per cent saline. The optimum quantity of polysaccharide for sensitization was determined by titrating batches of cells which had been sensitized with various quantities of alkali-treated LPS against the homologous S.gallinarum antiserum.

(c) HA and Antiglobulin HA tests: In the titration of antibody a 1.5 per cent suspension of sensitized erythrocytes was added to serial two-fold dilutions of the serum. The serum samples were obtained daily, inactivated by heating at 56°C for 30 minutes, and after a 5-fold dilution in saline they were assayed for antibody levels/

levels by the method of Buxton (1959_a), except that 0.2 ml. volumes were employed.

(d) The Specificity of LPS extract used in the HA Test: To ensure that the extract used to sensitise the chicken erythrocytes was specific and did not contain cross-reacting constituents, a series of cross haemagglutination tests were carried out: batches of cells ~~were~~ sensitized with each bacterial LPS extract (S.gallinarum and E.coli), and titrated against the homologous and heterologous antisera prepared in rabbits, and which had been absorbed with packed normal chicken erythrocytes.

The results of these experiments are shown in Table 5, which demonstrates conclusively the absence of any cross-haemagglutination between the antisera to S.gallinarum and E.coli of mixed serotypes.

Detection and Titration of in-vivo Sensitized Erythrocytes: The indirect and direct Coombs' test (ICT and DCT respectively) were employed for the detection and estimation of the presence and degree of erythrocyte sensitization which occurred during infection. These tests have been described in the previous chapter.

Sephadex G200 Chromatography: 1.5 mls. of the immune serum were fractionated at room temperature (18°C) on a column of Sephadex G200 (Pharmacia) measuring 60 cm. x 3 cm. after overnight equilibration against a buffer solution which contained 1 M NaCl in 0.1 M Tris, (pH 8.). The serum sample was put at the top of the column and a constant downward flow was maintained by a peristaltic pump. 5 ml. fractions were collected in a LKB Radirac fraction collector with time-controlled shift. Protein concentrations were automatically monitored by ultra-violet absorption at 280 mμ. The γ-macroglobulins (19S)/

TABLE 5
The Specificity of Purified S. gallinarum-Polysaccharide
Extract Used in the Haemagglutination (HA) Test.

SENSITIZING POLYSACCHARIDE EXTRACT	ABSORBED SPECIFIC RABBIT ANTI-O SERUM	
	SALM. GALLINARUM	E. COLI
SALM. GALLINARUM	1280*	O
E. COLI	O	320*
*= RECIPROCAL OF HAEMAGGLUTINATION TITRE		

(TABLE 5) DEMONSTRATES THE COMPLETE ABSENCE OF ANY CROSS HAEMAGGLUTINATION BETWEEN THE ANTISERA TO SALMONELLA GALLINARUM AND E. COLI (MIXED SEROTYPES).

γ -macroglobulins (19S) pass through quickly but the 7S globulins are delayed in their passage. The peaks and troughs were pooled into 12 fractions, dialysed against distilled water at 4°C for at least 72 hours and brought to the original volume by dialysis and ultrafiltration.

The fractions were assayed for specific O-antibodies by the HA and antiglobulin HA tests.










Micro-immunoelectrophoresis: Fractions were also examined by immunoelectrophoresis on microscope slides, using the modified micro-method described by Scheidegger (1955). Electrophoresis was carried out in 1 per cent Difco Noble agar gel in half-strength (ionic strength 0.05) barbitone acetate buffer (Oxoid), using a Kohn bath and a Vokam power pack. A potential of 110 volts (25m A) was applied for 4 hours and a rabbit anti-chicken whole serum was used for development after electrophoresis. Preparations were stained with Amidoschwartz.

RESULTS

(1) Development of Anaemia:

Further confirmation that chickens fatally infected with fowl typhoid develop a severe haemolytic anaemia (Chapter II) was obtained with the results recorded in Figs.10 - 16. There was also evidence of a strong compensatory response by individual chickens, and this was illustrated by the increase in the total number of reticulocytes and myeloblasts which appeared coincidentally with the development of anaemia. The results also indicated that in spite of these attempts by the hyperplastic bone marrow to compensate/

KEY FOR FIGURES 10 - 16.

	Total R.B.C. (T.R.B.C) - $\times 10^6/\text{mm}^3$
	Haematocrit (P.C.V.) - %
	Haemoglobin (Hb) - gms/100 ml
	Reticulocyte
	Antiglobulin titre
	Haemagglutinin titre
	Positive Indirect Coombs test (I.C.T.)
	Direct " "
	Died

Positive Indirect Coombs test (I.C.T.) } In-vivo
 " " " " } Erythrocyte
 Direct " " (D.C.T.) } Sensitization

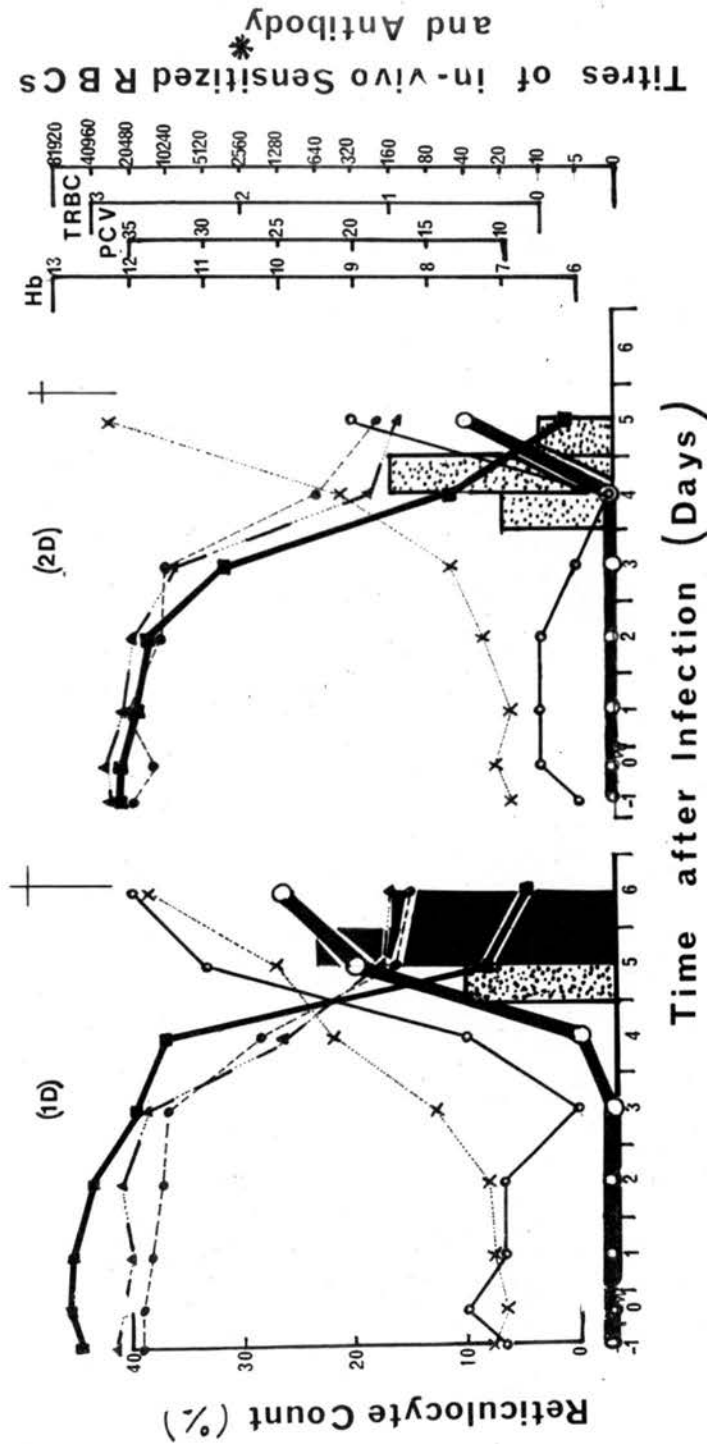


FIGURE 10. Haematological and Immunological Responses to Acute *S. gallinarum* Infection in the Chicken.

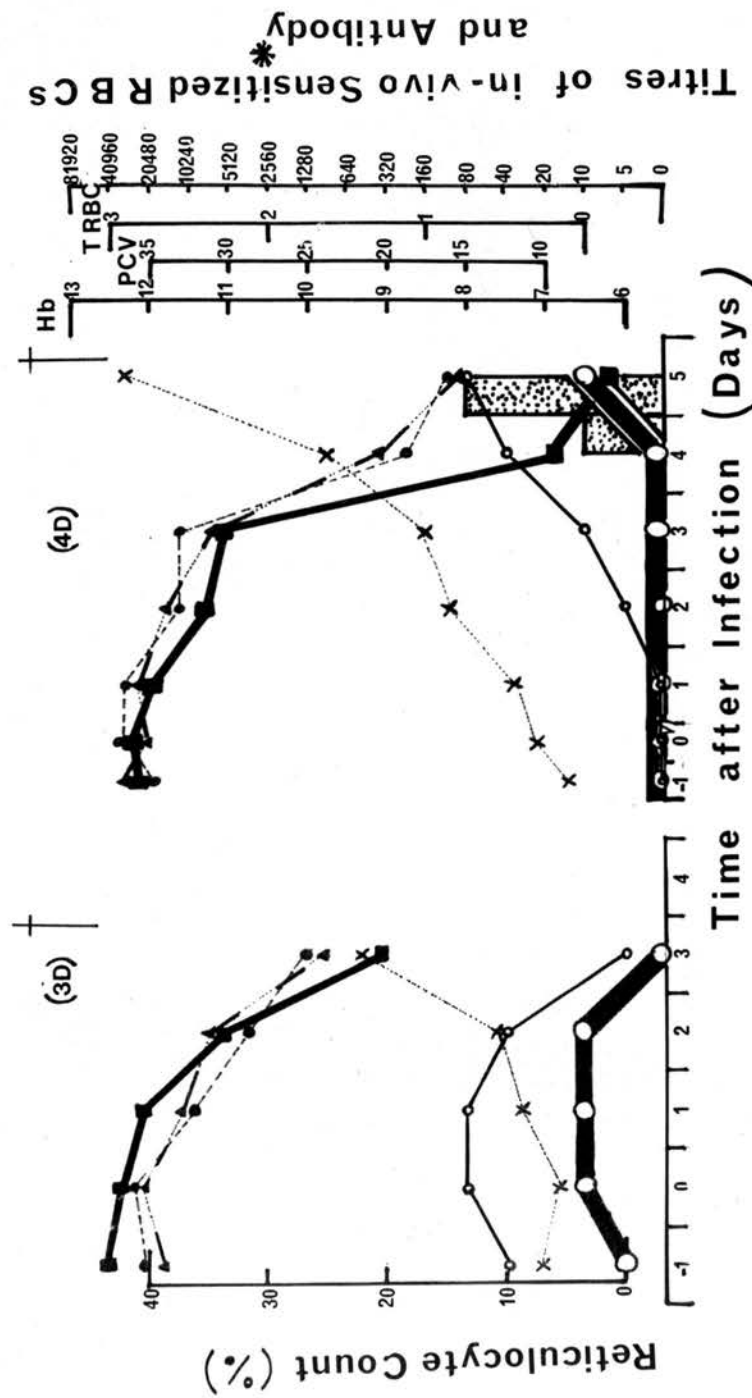


FIGURE 11. Haematological and Immunological Responses to Acute *S. gallinarum* Infection in the Chicken.

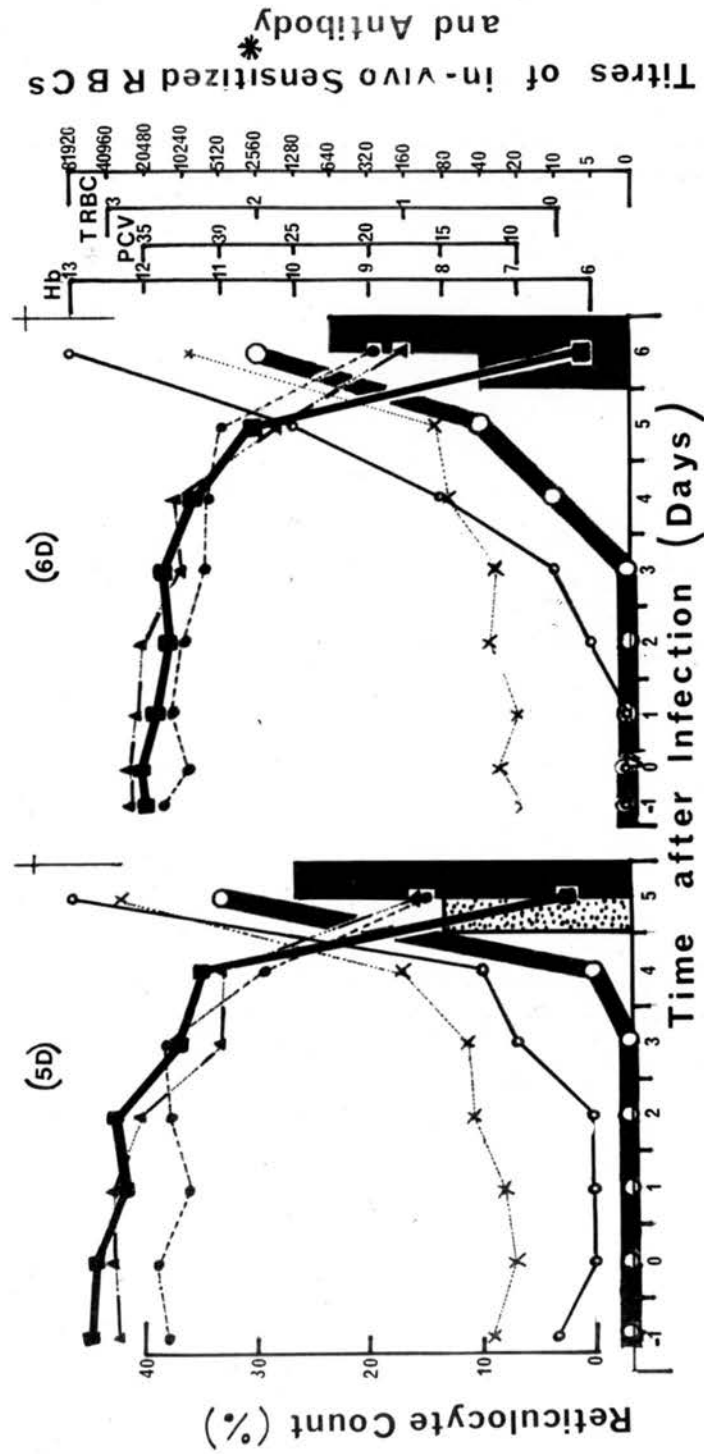


FIGURE 12. Haematological and Immunological Responses to Acute *S. gallinarum* Infection in the Chicken.

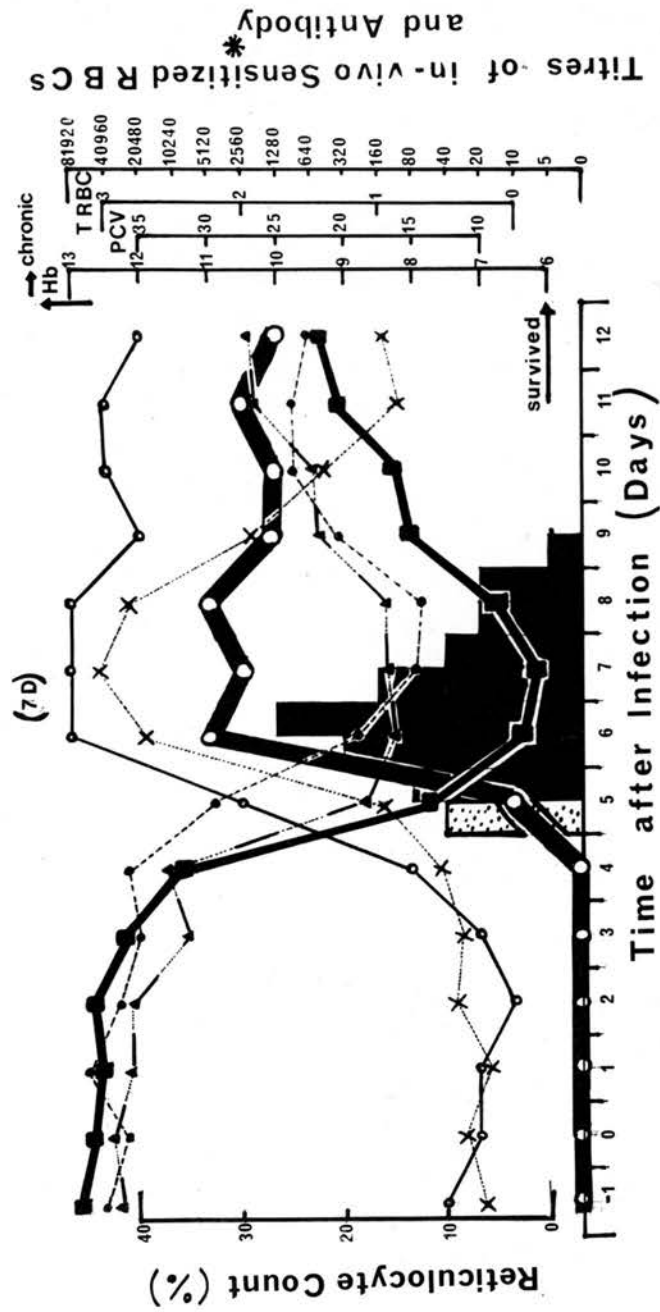


FIGURE 13. Haematological and Immunological Responses to Acute *S. gallinarum* Infection in the Chicken.

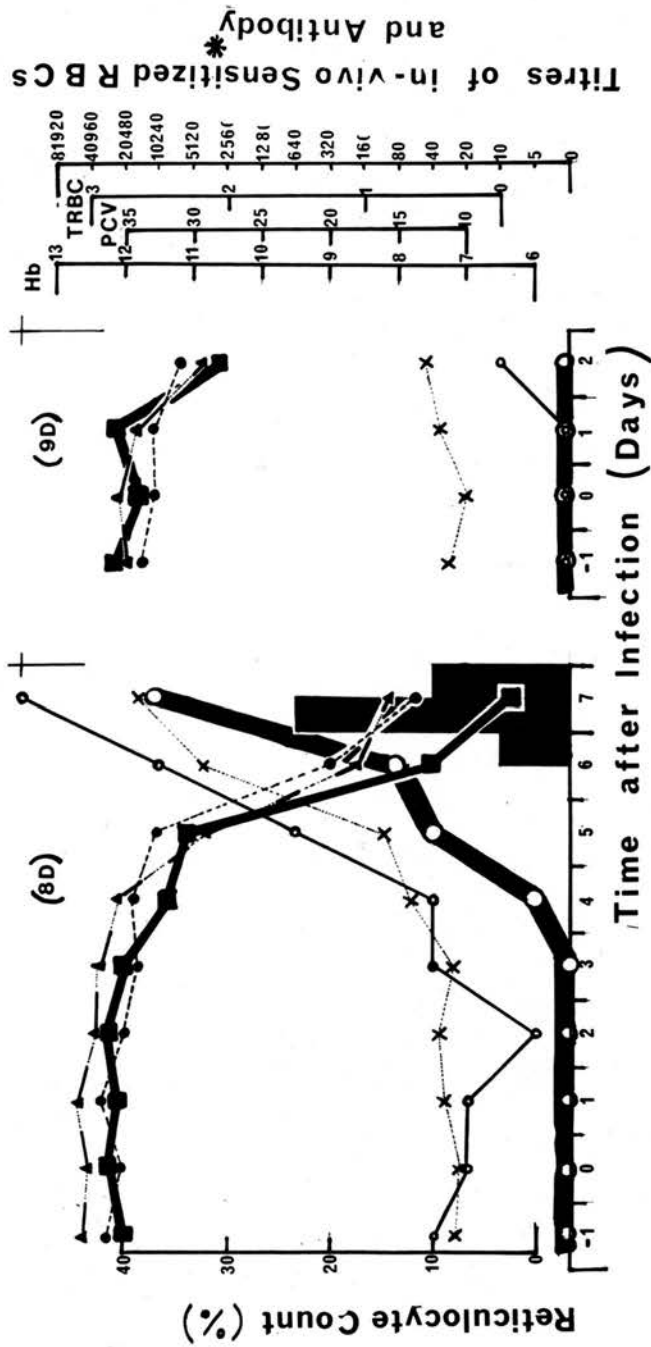


FIGURE 14. Haematological and Immunological Responses to Acute *S. gallinarum* Infection in the Chicken.

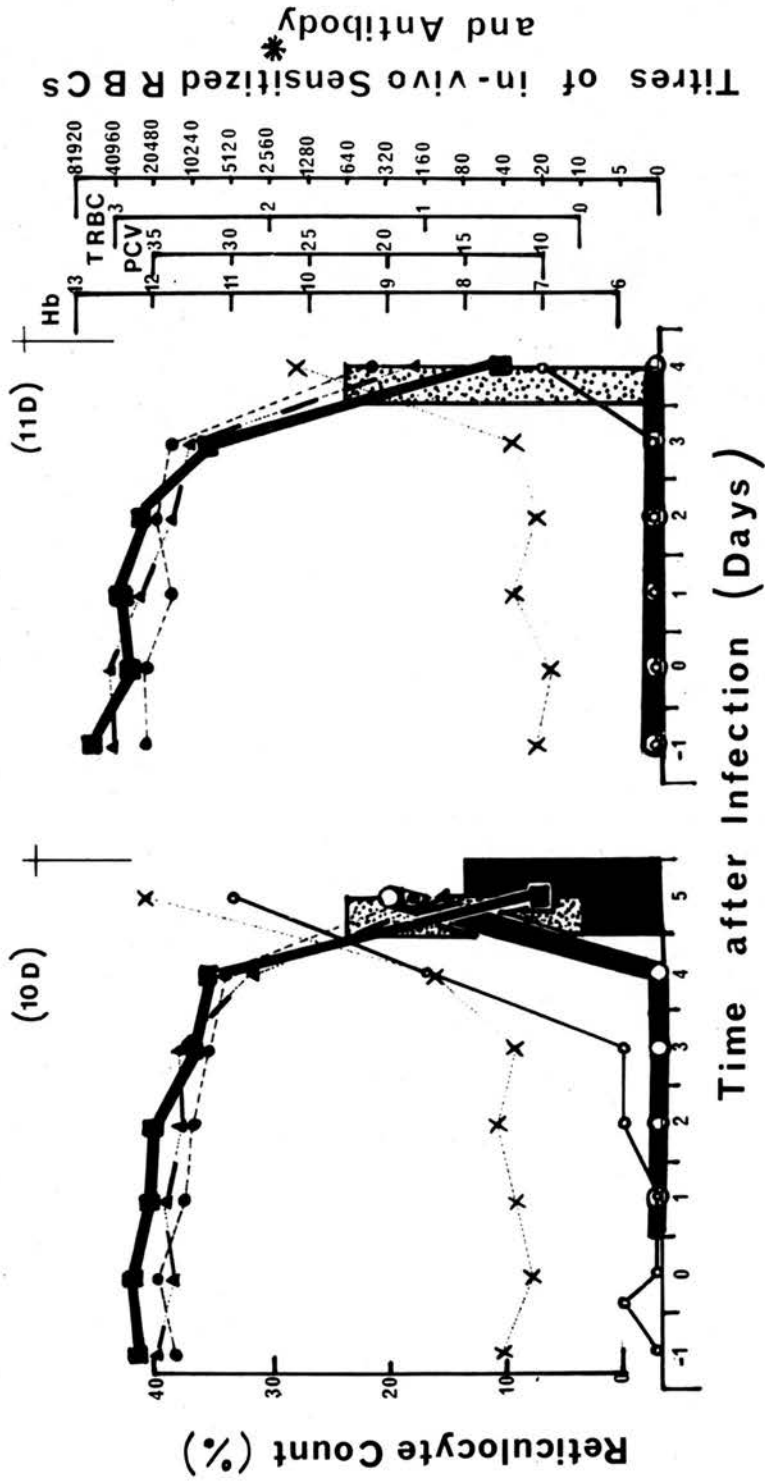


FIGURE 15. Haematological and Immunological Responses to Acute *S. gallinarum* Infection in the Chicken.

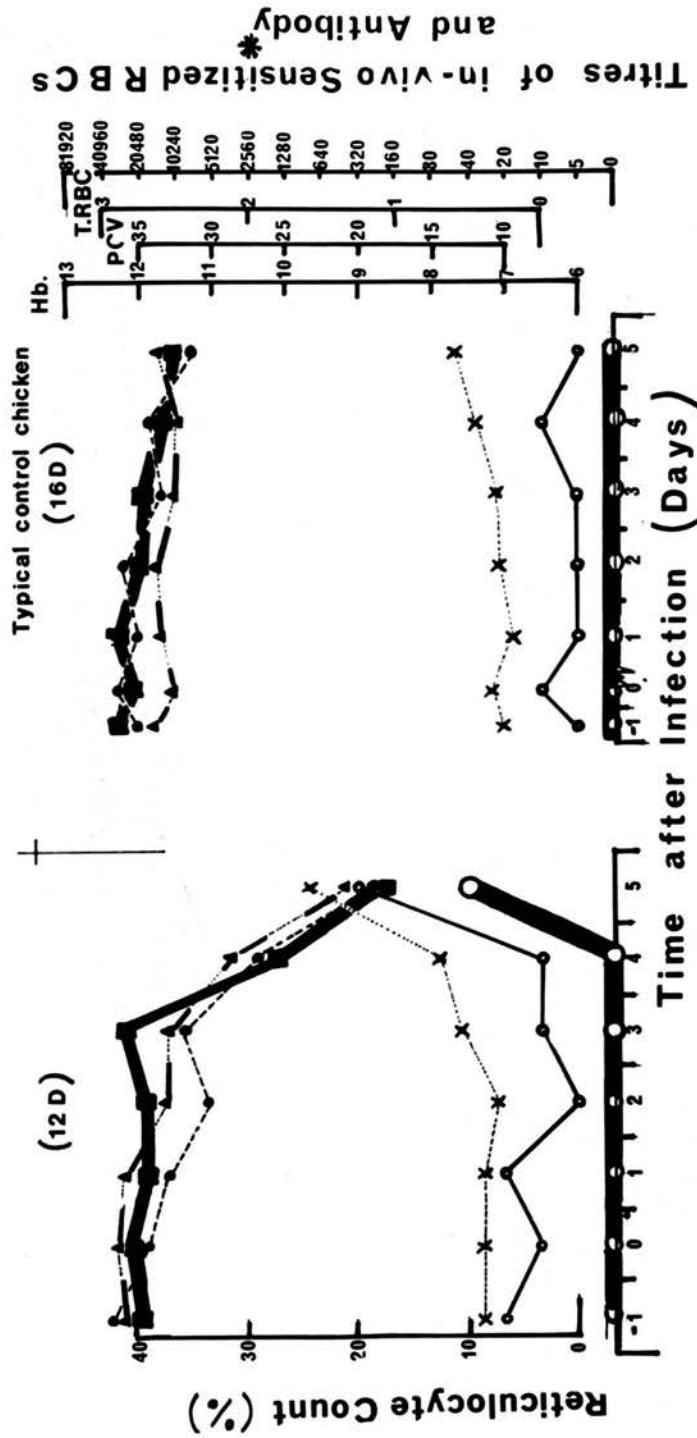


FIGURE 16. Haematological and Immunological Responses to Acute *S. gallinarum* Infection in the Chicken.

compensate for the severe loss, the rate of erythrocyte destruction greatly exceeded that of production of new cells, and a clinical anaemia was consequently observed. Only 1 out of the 12 infected chickens survived acute infection. Initially this chicken (7D) showed a reduction in haemoglobin, PCV and total circulating erythrocyte levels comparable with the other infected chickens, but by the eighth day after infection these levels had begun to return to normal and it eventually became clinically normal between the fourteenth and sixteenth days following infection (Fig.13). This particular animal died a few weeks later with lesions of pericarditis. However, the haematologic values remained constantly at sub-normal levels.

In view of the finding by Buxton (1960) that circulating erythrocytes develop an abnormal osmotic fragility during the acute stage of the disease, and the coincidence of the terminal stages of infection with both in-vivo erythrocyte sensitization and the development of a haemolytic anaemia in the present studies (Chapter II), it was considered that erythrocyte fragility tests performed at this time might show significant variations from the normal which could be related to the pathogenesis of the infection.

(2) Changes in Resistance of Erythrocytes to Haemolysis in Hypotonic Salt Solutions during Infection:

To obtain more information on the osmotic fragility tests, preliminary experiments were performed on blood from normal chickens. The results obtained showed that the fragility of normal nucleated avian erythrocytes was not dissimilar from that of non-nucleated mammalian erythrocytes (Dacie and Lewis, 1968). Maximum haemolysis/

haemolysis with avian erythrocytes occurred at salt concentrations of 0.4 to 0.45 per cent, but with the majority of normal chickens the range of salt concentrations within which any degree of haemolysis occurred was between 0.30 and 0.55 per cent, with occasional "tails" at either of these limits of salt concentrations. This range of figures for haemolysis was slightly more widespread than Buxton's previous figures of 0.35 to 0.40 per cent (Buxton, 1960).

The characteristics of osmotic fragility curves obtained from 4 of the infected chickens, representing the various "types" of erythrocyte sensitization (vide infra), are shown in Figs. 17 and 18. It will be noticed that in all 3 positive sensitization types (II, III and IV) (vide infra and Fig. 19) the normal curves for erythrocyte fragility became replaced by binodal curves which are characteristic of haemorrhage or haemolysis in mammals (Suess et al., 1948; Bolton, 1949), and birds (Buxton, 1960). The earliest appearance of these curves was a few hours before death, and it is of interest to note that this coincided with both the time at which sensitized erythrocytes were detected and the development of the haemolytic anaemia. It is significant that where there was no detectable sensitization of erythrocytes there was no occurrence of binodal fragility (See chicken nos. 3D and 16D of Figs. 17 and 18 respectively).

These osmotic fragility curves illustrate clearly that during haemolytic anaemia the peaks of the curves recording fragility show a distinct "shift to the right", the binodal curve reflecting the presence of 2 cell populations of different susceptibilities to hypotonic NaCl solutions; part of the cell population clearly undergoes haemolysis at a higher salt concentration/

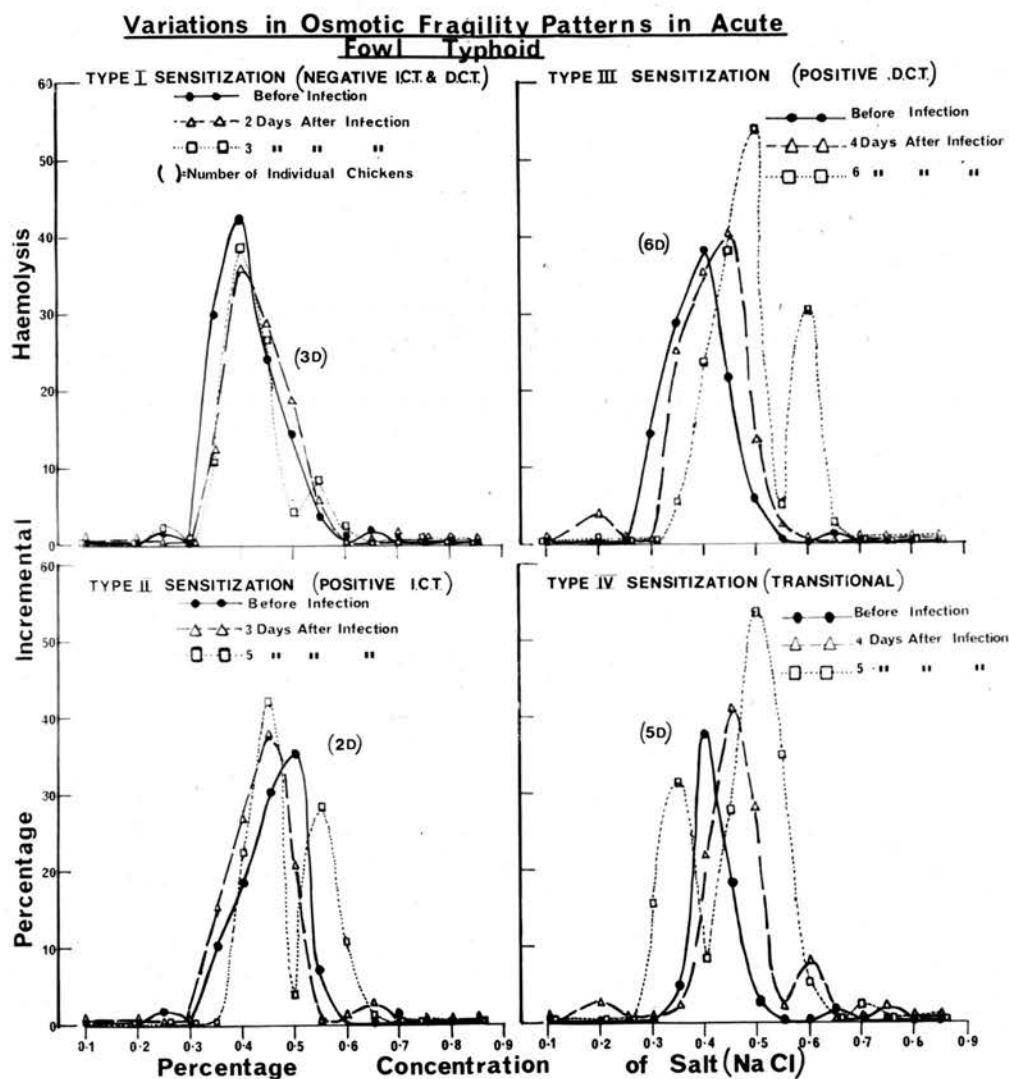


FIGURE 17. Changes in Resistance of Erythrocytes to Haemolysis in Hypotonic Salt Solutions During Fowl Typhoid. Single osmotic fragility curves of normal erythrocytes replaced by binodal curves when erythrocytes became sensitized in vivo (See Figs. 10 - 12).

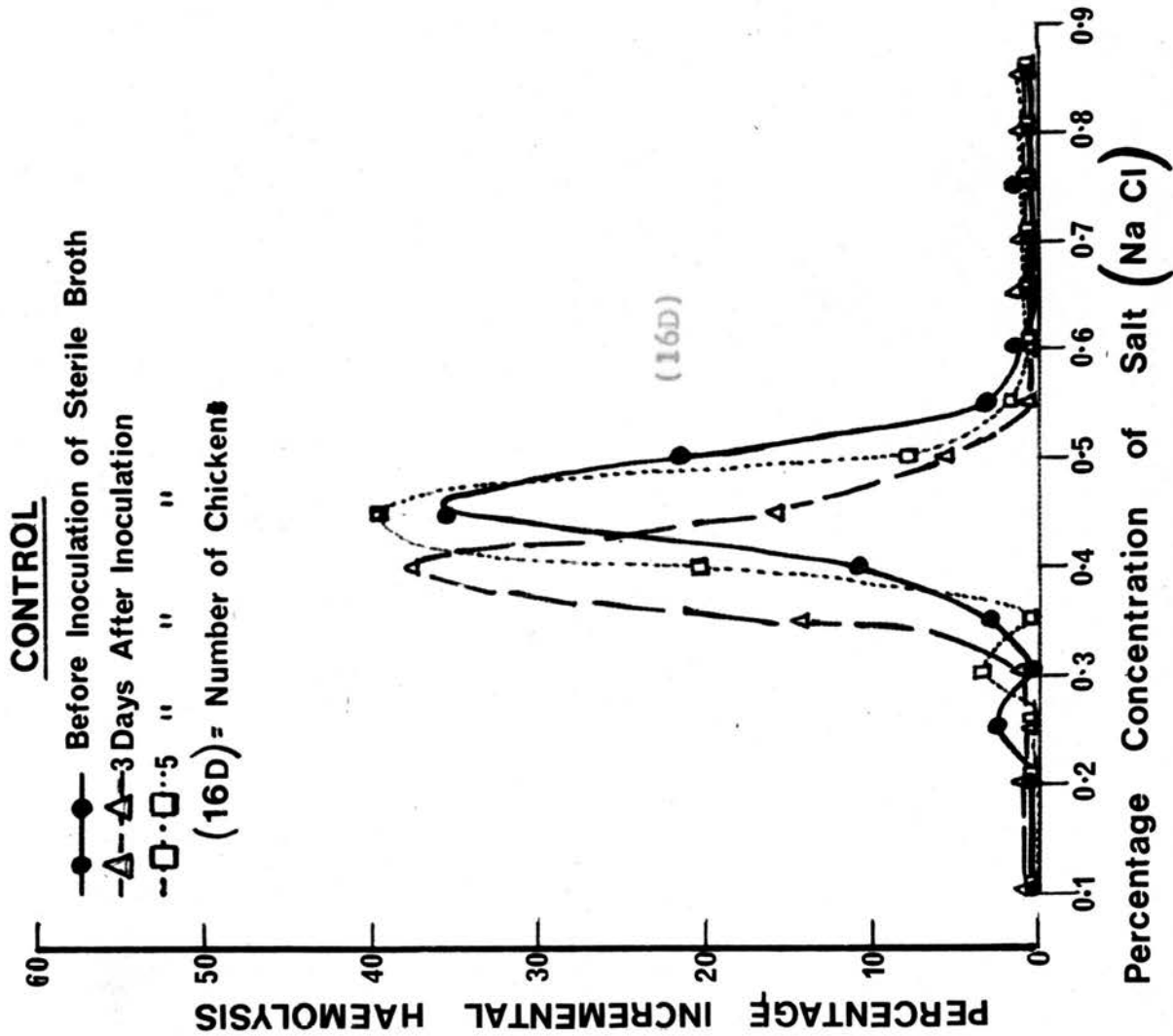


FIGURE 18. Changes in Osmotic Fragility Curves in Control Chicken (16D).
 Note absence of detectable in-vivo erythrocyte sensitization
 and no occurrence of binodal fragility curves (See also
 Chicken 3D in Fig. 17).

concentration, between 0.55 and 0.65 per cent. It is suggested that the increased fragility may be due to the decreased resistance of the sensitized or altered erythrocytes in hypotonic salt solutions.

In the only chicken that survived (7D in Fig.13), it was observed that the second curve which had appeared on the sixth day had completely disappeared by the ninth day — the same day that sensitized cells could no longer be detected. This observation, coupled with the finding that death of the other chickens which showed erythrocyte sensitization had occurred soon after the disappearance of this abnormal fragility, suggest that these phenomena are intimately associated with a critical stage of infection. Moreover, the temporary nature of the binodal curve obtained from the surviving chicken, lends support to the possibility that the abnormal fragility of erythrocytes may have resulted from adsorption of bacterial antigen on to their surfaces during infection; and that a direct relationship may exist between the pattern of osmotic fragility, erythrocyte sensitization and infection with S.gallinarum as previously suggested (Buxton, 1960).

(3) In-vivo Sensitization of Erythrocytes During Infection

(a) Classification: Throughout the present study, and in subsequent chapters, it was observed that there were distinct variations in the way in which the sensitized cells were being detected by the ICT and DCT. For example, some of the cells could only be detected with the ICT, presumably because of an adsorbed bacterial antigen. Other cells were also positive to the DCT, indicating the presence of adsorbed serum globulins, possibly as
a/

a result of pre-adsorption of bacterial antigen. These varying erythrocyte surface alterations, occurring during infection, offered the possibility that different 'types' of erythrocyte sensitization may exist in this disease. Inspection of infection parameters of individual chickens (Figs. 10 to 16) reveals that at least 4 sensitization patterns are present. These types of sensitization are illustrated diagrammatically in Fig.19, which shows 3 positive types and one negative (non-sensitized) type:

- List of Types: I — Negative ICT and DCT (No sensitization).
 II — Positive ICT.
 III — Positive DCT.
 IV — Positive ICT later becomes Positive to DCT (Transitional).

(b) Pattern of In-vivo Sensitization: Chicken numbers 3D, 9D and 12D (Figs. 11, 14 and 16) and 30H and 33H (Table 3 in Chapter II) showed no sensitization of their erythrocytes (Type I) within the limits of sensitivity of the tests employed. Such animals invariably died in the per-acute stage and, on average, within 3 days of infection. In general, however, no animals died at peak time — between 4 and 6 days — without exhibiting erythrocyte sensitization (Fig.21), even though, as shown later (vide infra), there were a few cases of chickens showing erythrocyte sensitization but surviving (Table 8); Table 8 also shows that only 3 chickens had survived infection and shown no detectable sensitization.

The 3 positive types of erythrocyte sensitization, classified/

TYPES OF IN-VIVO ERYTHROCYTE SENSITIZATION




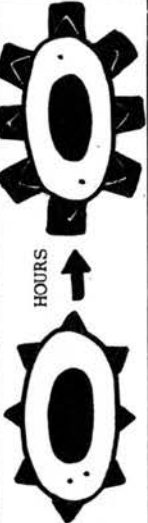


TYPE OF SENSITIZATION	METHOD OF DETECTION OF SENSITIZATION	MODE OF ERYTHROCYTE SENSITIZATION	DIAGRAMMATIC ILLUSTRATION
I	INDIRECT AND DIRECT COOMBS TESTS (ICT and DCT)	a) <u>Negative ICT and DCT</u> b) No Sensitization (Probably dependent upon the sensitivity of tests used for detection)	
II	INDIRECT COOMBS' TEST (ICT)	a) <u>Positive ICT</u> b) Antigen (? lipopolysaccharide) which has become adsorbed on to erythrocyte surface.	
III	DIRECT COOMBS' TEST (DCT)	a) <u>Positive DCT</u> b) This 'antigen-antibody' complex is detected on cell surface without prior observation of adsorbed antigen. c) Immune globulins directed against hitherto masked (intrinsic) surface antigens also give +ve DCT.	
IV	(TRANSITIONAL) ICT and DCT	a) <u>Positive ICT and DCT</u> b) Probably homologous antibody reacting later with antigen which had become attached to erythrocyte surface.	
<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">  <u>ANTIGENS</u> (?LIPOLYSACCHARIDE) </div> <div style="text-align: center;">  <u>ANTIBODY</u> </div> </div>			

FIGURE 19. Diagrammatic Illustration of the Four Types of in-vivo Erythrocyte Sensitization.

classified here as Type II, III and IV, are also demonstrated in individual chickens by Figs. 10-15. Type II sensitization refers to erythrocytes which have probably adsorbed a bacterial antigen in-vivo, since they agglutinate only with the indirect Coombs' test (ICT). This type is illustrated by chicken numbers 2D, 4D and 11D in Figs. 10, 11 and 15.

Another type of sensitization, referred to as Type III, occurs when they are detected by a positive reaction to a direct Coombs' test (DCT). Chicken numbers 6D and 8D of Figs. 12 and 14, illustrate this type of in-vivo erythrocyte sensitization. The ICT was never positive prior to being directly agglutinated by the DCT. In contrast, cells referred to as the Type IV, became positive to the ICT, and later were observed to be transformed because they became agglutinable by the DCT. Type IV sensitization is also referred to as the "Transitional" type. Examples of this are illustrated by chickens 1D, 5D, 7D and 10D in Figs. 10, 12, 13 and 15. Both Types III and IV sensitized cells occur mainly at the peak of infection and mortality, but only Type III cells are detected later on during infection (Fig. 21).

An interesting observation, on the Type II pattern of sensitization (positive ICT) throughout these studies, was the rapid disappearance of such cells from the circulation, and this usually occurred a few hours before death (Table 6). In a number of chickens where the duration of these cells in the circulation was timed, none of them persisted for more than 36 hours (Table 6), and in one of the animals (42H) the sensitized cells had completely disappeared from its circulation within 6 hours of first detection.

Another intriguing observation was the finding that/

TABLE 6

DURATION OF TYPE II SENSITIZED (POSITIVE ICT) RBC^a IN THE
CIRCULATION

CHICKEN No.	DURATION OF SENS. RBC IN CIRCULATION (in hours)
3B	15
6B	24
2C	4
4C	36
7C	21
9C	18
2D	36
4D	24
11D	8
5S	24
4R	10
38H	5
40H	9
42H	6

TABLE 7TYPE IV SENSITIZATION: TIME OF TRANSITION OF SOME FATAL CASES

CHICKEN No.	TIME OF TRANSITION FROM TYPE II TO III (In hours)
1B	8
5B	13
3C	18
8C	14
10C	28
1D	4
5D	7
7D	11
10D	3
2R	8
31H	15
36H	26
37H	11
41H	6

that the time of transition of Type II to Type III (that is, the Type IV sensitization), was relatively short; it barely exceeded 24 hours (Table 7). These animals were timed from the period when their erythrocytes first became positive against the ICT and when these cells later could be directly agglutinated with the anti-globulin serum (DCT). The longest recorded period of transition in the circulation was 28 hours (chicken 10C), but as short as 3 hours transitional time was recorded for chicken 10D.

In the experiments reported above, it was noticed that erythrocyte sensitization always occurred at the time of maximum haemolytic activity (Figs. 10-15), and invariably a few hours before death. It was observed that in those chickens (2D, 4D and 11D of Figs. 10, 11 and 15) exhibiting Type II sensitization, the sensitized cells could no longer be detected a few minutes before death. This effect was not observed with the other types of sensitization, where some of the sensitized cells could still be detected before death.

(c) Frequency of in-vivo Erythrocyte Sensitization During Infection: When groups of infected animals, which had been examined throughout the present thesis for sensitization of their erythrocytes, were collected and analysed, some interesting findings were made. The results of this analysis, summarised in Table 8, clearly demonstrate that in-vivo sensitization of erythrocytes is a relatively common phenomenon in this disease syndrome. 78 out of 93 infected chickens died, and of those that died, nearly 85 per cent of them had exhibited one type or other of positive erythrocyte sensitization. In contrast, only 15.4 per cent of fatal cases did not/

not show any sensitization of their erythrocytes. The highest number of chickens that died were demonstrated to have had a Type III sensitization (32.1 per cent) followed by Types IV, II and I, in that order of decreasing incidence. These observations suggest a closer relationship between in-vivo sensitization and rate of mortality than has hitherto been demonstrated.

(4) The Immunological Response to Infection:

Observations above have shown that the haemolytic episode associated with acute fowl typhoid generally occurs between, the fourth and seventh days after infection; and this period also nearly always coincided with the time of detection of erythrocyte sensitization. It was also observed that some of the sensitized cells (Type III) were directly agglutinated by the antiglobulin serum, indicating that globulins, probably of antibody nature, may have been adsorbed in-vivo. It was therefore decided to investigate whether specific antibody response can be mounted in such a short time during the acute infection. Such a study, of the active immune response in relation to other haematological parameters during the acute disease, would be of great value in elucidating any possible immunological basis of the mechanism of this anaemia.

(a) Natural Antibodies in Adult Normal Chicken Sera: Sera obtained from chickens before infection were occasionally found to contain antiglobulin haemagglutinating antibodies to the O-antigen of S.gallinarum (See Figs.10 - 16). These animals had been obtained from a commercial flock known to be free from typhoid infection and unvaccinated. These antibodies may therefore be cross-agglutinating, the result of earlier antigenic stimulation by antigens possessing related/

TABLE 8

THE RELATIONSHIP BETWEEN IN-VIVO ERYTHROCYTE SENSITIZATION AND DISEASE

GROUP OF CHICKENS	TOTAL NUMBER OF CHICKENS	TYPE OF ERYTHROCYTE SENSITIZATION				NUMBER OF CHICKENS SHOWING ERYTHROCYTE SENSITIZATION (%)
		I (-ive DCT & ICT)	II (+ive ICT)	III (+ive DCT)	IV (TRANSITIONAL) (+ive ICT to +ive DCT)	
TOTAL	93	15	22	29	27	83.9
DIED	78	12	19	25	22	84.6
SURVIVED	15	3	3	4	5	80
CONTROLS	48	48	0	0	0	0

related determinants to the O-antigens (1, 9 and 12) of S.gallinarum.

(b) Immune Response During Infection: The variations and heterogeneity in the antibodies to S.gallinarum as measured by the haemagglutination (HA) and antiglobulin HA tests, and their relationships to the haematologic response during infection, are illustrated in Figs.10 - 16. They show that in chickens in which haemagglutinating antibodies are detected at an early stage, Types III and IV erythrocyte sensitization were invariably found. This is illustrated by chicken numbers 1D, 5D, 6D and 8D in Figs. 10, 12 and 14. In contrast, where the haemagglutinating type of antibody is delayed in appearance, Type II sensitized cells were only detected; for example in chicken nos. 2D, 4D and 11D (Figs.10, 11 and 15). Furthermore, this type of sensitization was also seen when haemagglutinating antibodies were not detected (11D in Fig.15).

It was also observed that there was an initial decline in the mean antiglobulin HA titre during infection in cases where a significant pre-infection incomplete antibody titre (between 1:20 and 1:80) had been demonstrated (See chicken nos. 1D, 2D, 3D, 8D and 12D — Figs. 10,11,14 and 14). In animals where no natural antibodies had been detected before infection, the earliest time of demonstrating antiglobulin haemagglutinins was 2 days after infection (See chicken nos. 4D, 6D, 9D and 10D). On the other hand, the earliest detectable complete haemagglutinins developed between the fourth and fifth days after infection; (that is, all chickens except 3D, 9D and 11D).

Inspection of Figs. 10 - 16 again reveals that the time/

time of appearance of haemagglutinins, especially of the complete type, seems to be significant in relation to the haemolytic response during infection. The coincidence of these factors strongly suggested that immune response may be involved in the development of the haemolytic anaemia.

(c) Distribution of Antibodies against *S.gallinarum* Lipopolysaccharide in Immune Serum Fractions (5D/5/67): It was also decided to study the character and class of the antibody which may be involved in the elimination and destruction of in-vivo sensitized erythrocytes. For this purpose immune sera, taken during the critical peak periods of the haemolytic episode, were fractionated, using Sephadex G200.

The elution pattern, pooling data, the immunoelectrophoretograms, haemagglutinating titrations, and the distribution of protein and haemagglutinins in the pooled fractions of one of these immune serum samples (5D/5/67) are given in Fig. 20a, b. It can be seen from the serum profile that protein was eluted from the column in 3 peaks. In order of elution these have been shown to comprise 19S and 7S and 4.5S proteins respectively (Flodin and Killander, 1962). Complete haemagglutinating antibodies were detected only in fractions constituting the 19S peak, but small amounts of the non-haemagglutinating antibody could be detected in this peak also. Specific IgG antibody was mainly present in the second peak, and could be detected only by the antiglobulin HA test. Small amounts of these non-haemagglutinating antibodies were also found in the third peak.

Fractionation of other immune serum samples obtained from infected chickens just before death, and at the peak of the anaemias, yielded similar results.

Digestion/

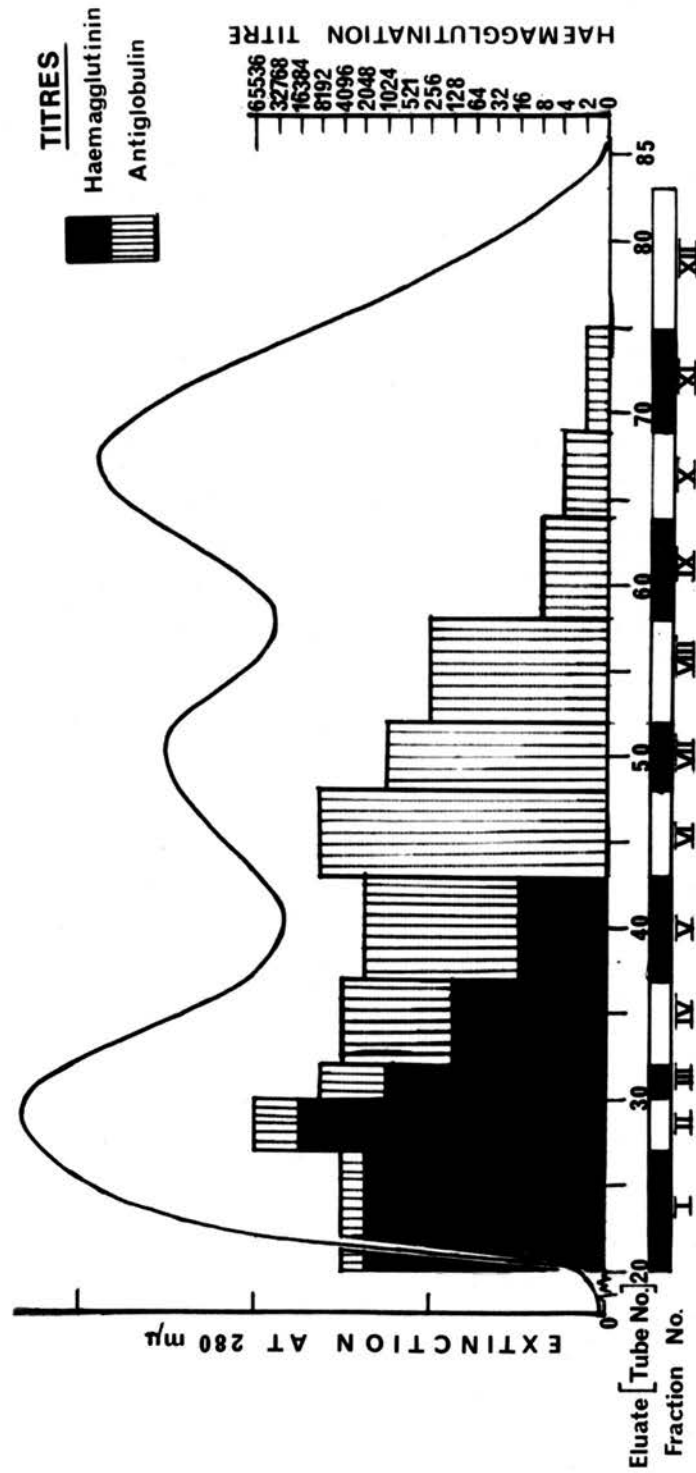


FIGURE 20a. Sephadex G-200 Fractionation of an Immune Serum 5D/5/67. Showing the elution pattern, pooling data, haemagglutinating titrations and distribution of protein and antibodies against *S.gallinarum* polysaccharide in the pooled fractions.

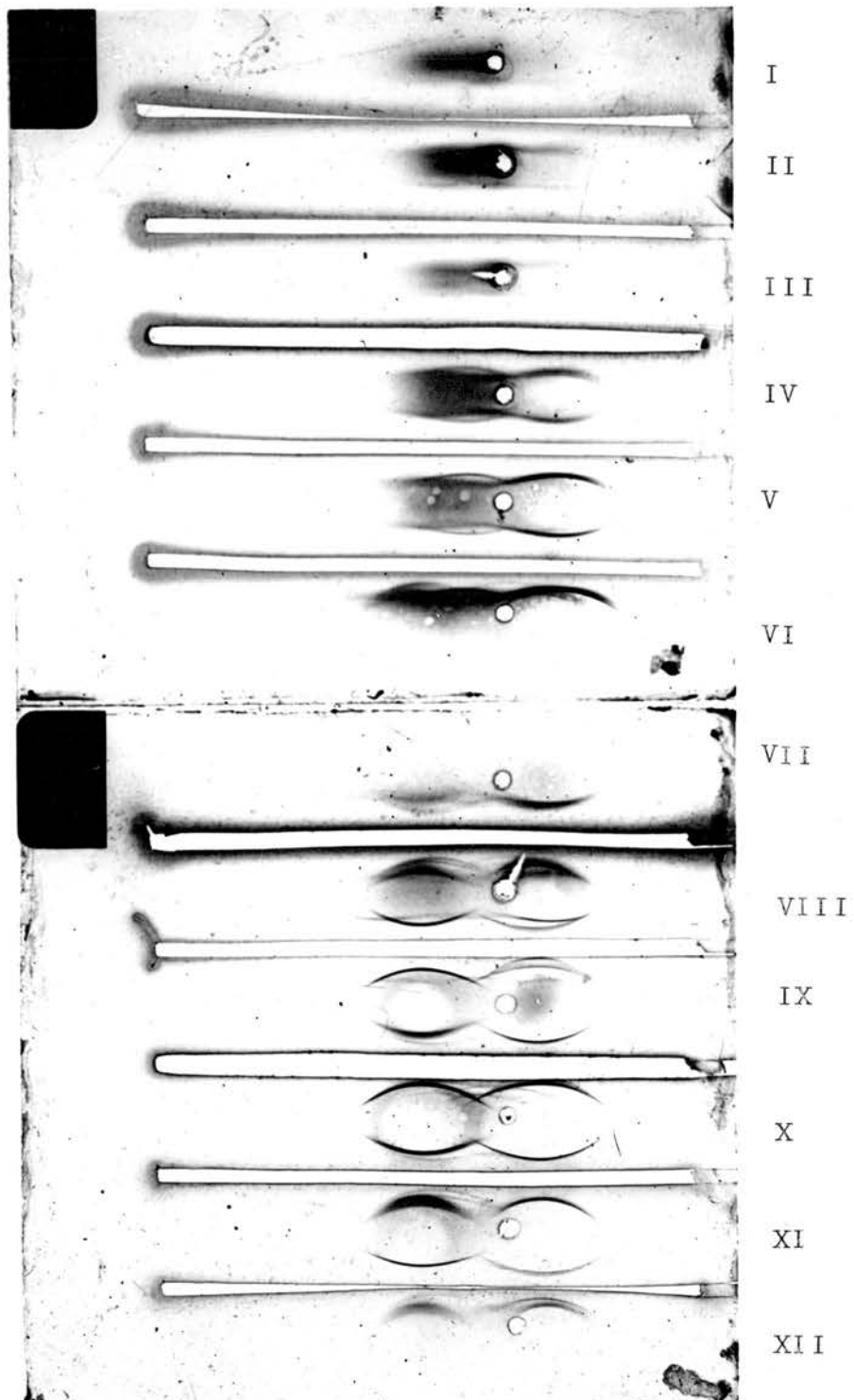


FIGURE 2Ob. The Immuno-electrophoretograms of the Pooled Fractions (I - XII) of Immune Serum 5D/5/67. (Anode to the Left).

Digestion of macroglobulin antibodies and resistance to inactivation of 7S antibodies by 2-mercaptoethanol (2-ME) has been widely used as a means of distinguishing IgM from IgG (Deutsch and Morton, 1957; Grubb and Swahn, 1958). In the present studies, partial inactivation of the IgG antibodies was obtained with the fractionated chicken sera. This finding was not surprising in view of earlier findings that partial IgG inactivation with 2-ME occurs with chicken antibody (Rosenquist and Gilden, 1963; Szenberg, Lind and Clarke, 1965).

DISCUSSION

It is evident from the above studies, as well as from those of the previous chapter, that the phenomenon of in-vivo sensitization of erythrocytes during acute fowl typhoid is of a common occurrence (Table 8) and is also associated with a critical stage of the induction of the anaemia and the disease syndrome itself.

The hypothesis has been offered above which suggests that during infection the attachment of bacterial antigen to erythrocytes in-vivo subsequently results in the rapid elimination and destruction of these sensitized erythrocytes as a consequence of their reaction with homologous bacterial antibodies. The view has, however, been expressed elsewhere that since in-vivo erythrocyte sensitization is a rare and uncommon event in bacterial infections in general, the phenomenon therefore may play only a minor role in the pathogenesis of anaemia of these infections (Shumway, Bokkenheuser, Pollock and Neter, 1963; Dacie, 1967). This assumption, and the reasoning behind it, appears to be incorrect/

incorrect for two reasons. Firstly, as reviewed in Chapter I, in-vivo sensitization is not uncommon; it may occur during many bacterial, especially enteric infections, and may not have been detected in the past for the simple reason that it was not sought. In the past, anaemia of acute infection was regarded as the dyshaemopoietic type (Saifi and Vaughan, 1944; Cartwright and Wintrobe, 1952), and it was believed that the disturbance of erythropoiesis is merely part of the general toxæmia or disturbance of metabolism. Consequently, not often were the serological modifications and the immune responses examined. Besides, careful inspection of the data presented by various workers who had reported the occurrence of in-vivo erythrocyte serological modification during certain gram-negative bacterial infections (Berman, Brown and Rahmilewitz, 1945; Shaw, 1951; McFadzean and Choa, 1953; Grobbelaar, 1958; Ruggieri, 1961; Young et al., 1962; Retief and Hofmeyr, 1965) reveals that the detection of even some of these erythrocyte alterations has been fortuitous. Since the phenomenon of in-vivo sensitization frequently occurs in the absence of underlying haemolytic anaemia (Stratton and Renton, 1959; Saito et al., 1961; Young et al., 1962; Springer and Ansell, 1962; Springer and Horton, 1964) it is possible that the detection of sensitization generally would be left to accident, except for those cases showing overt haemolysis. Consequently the observed incidence of in-vivo sensitization in bacterial infections would be on the low side. Furthermore, the scarcity of published reports on erythrocyte changes in acute bacterial infections, especially those caused by gram-negative bacteria, may also be due to the fact that the associated anaemia is of uncommon occurrence (Cartwright/

Cartwright and Wintrobe, 1952; Weinstein and Beutler, 1962; Mitus, 1966; Editorial, 1967). The anaemia of acute gram-negative bacterial infections is probably commonly of the low-grade variety; in that case it is conceivable that not much attention would be paid to this clinical symptom unless it is severe enough to mask the other clinical signs.

The second reason is that there have been no studies to show unequivocally what role the phenomenon of in-vivo sensitization plays in the rapid destruction of these cells. It has been impossible, before now, to obtain an adequate and convincing direct evidence to support the hypothesis offered above, but as the present results indicate, fowl typhoid presents a suitable experimental model for this purpose.

The experiments carried out in the present study lend convincing support to the concept offered above for the mechanism of anaemia of acute fowl typhoid, and probably other gram-negative bacterial infections as well. Buxton (1960) has suggested that the development of the anaemia is the result of increased activity of the RES, which may become stimulated during S.gallinarum infection and therefore may be able increasingly to take up sensitized erythrocytes. Nevertheless, the present demonstration of coincidence of erythrocyte sensitization and the onset of haemolytic episode, with a sharp increase in the haemagglutinating antibody response, makes it more likely that the rapid destruction of erythrocytes is a consequence of an in-vivo antigen-antibody reaction, analogous to a Type II hypersensitive reaction described by Gell and Coombs (1968). This conclusion is supported by the finding of a sudden decrease in haematologic values — for example, haemoglobin/

haemoglobin, haematocrit, and total circulating erythrocytes — at a time when homologous bacterial antibodies, engendered during infection, appear in the circulation. These processes were also found to coincide with the period when in-vivo erythrocyte sensitization could be detected and, in some cases, sudden disappearance of these sensitized cells could be demonstrated during this critical period. The haemolytic anaemia which develops characteristically during this infection may therefore be the result of a specific immunological reaction.

The suggestion that the sensitized erythrocytes are rapidly removed and clearance is probably enhanced by the appearance of homologous bacterial antibodies is in agreement with the finding in other species that in-vivo haemagglutination, arising from antigen-antibody reactions, results in the rapid removal of the erythrocytes by the RES (Jandl, Jones and Castle, 1957; Mollison and Hughes-Jones, 1958). Furthermore, the finding that intravenously injected heterologous and homologous erythrocytes in animals possessing, respectively natural hetero- or iso-antibodies, are rapidly cleared from the circulation and destroyed by the RES (Benacerraf et al., 1957; Hughes-Jones et al., 1957; Jandl and Tomlinson, 1958; Cutbush and Mollison, 1958; Cutler, 1961; Mollison, 1962; Mollison and Hughes-Jones, 1967) lends further support to the contention that the anaemia of fowl typhoid may primarily be due to the alteration of erythrocytes or occurrence of immunological reactions on the erythrocyte surface and only secondarily to the stimulation of the RES (Chapter II).

Even though in-vivo erythrocyte sensitization has been shown by previous workers to be possible (Saito et al., 1961; Young et al., 1962; Springer and Ansell, 1962; Springer and Horton/

Horton, 1964; Springer, 1964), the occurrence of overt haemolytic anaemia was not reported. Nevertheless, numerous other clinical observations on tuberculosis in human and animal patients have demonstrated the association between tuberculosis and increased haemolysis. (Mollison, 1947; Lindeboom, 1950; Sandage, Brandt and Birkeland, 1951; Klimeš and Celer, 1960; Jandl, Jacob and Daland, 1961). Similarly, when Young and colleagues (Young et al., 1962) reported that erythrocytes from 2 infants with E.coli enteritis had acquired bacterial polysaccharides on their surfaces they did not report any concomittant development of anaemia. From their data they suggested that bacterial lipopolysaccharides, some of which had blood group B-like antigens, crossed the intestinal mucosa to enter the infants' circulation during attacks of diarrhoea. Nearly identical findings, where blood group B-like antigens or E.coli polysaccharides have been detected on erythrocytes of children and chickens with severe gastroenteritis, have been reported by Springer and Ansell (1962), Springer and Horton (1964) and Springer (1964). Isolated, soluble blood group B-antigens have since then been found to be serologically and antigenically identical with bacterial lipopolysaccharides (Springer and Ansell, 1962; Springer and Horton, 1964; Springer, Wang, Nichols and Shear, 1966; Springer, 1967).

The main interest, however, of the above authors in the clinical cases of in-vivo erythrocyte sensitization reported seemed to be merely concerned with the serologic and antigenic nature of the acquired erythrocyte antigen. Nevertheless, there are several recorded observations of the occurrence of abnormalities of erythrocytes and haemolytic episodes in man and animals with diverse/

diverse forms of severe enteritis, even though their association and probably significance have not been generally stressed. For example, severe haemolytic episodes and positive direct Coombs' test (DCT) are well-known complications of S.typhi infection in man (Flandin et al., 1935; Berman et al., 1945; Shaw, 1951; Gordon-Smith, 1951; McFadzean and Choa, 1953; Grobblelaar, 1958; Ruggieri, 1961; Retief and Hofmeyr, 1965) and other salmonella infections (Davidson and Fullerton, 1958; Dacie, 1967).

Presumably in these cases the positive antiglobulin tests were detecting anti-salmonella antibodies developed against adsorbed salmonella polysaccharide. This conclusion is supported by the present finding of similar abnormalities (Types III and IV sensitization) of erythrocytes during acute fowl typhoid. Furthermore, the finding that the time of transition from Type II to Type III sensitization generally did not exceed 24 hours (Table 7), may also help to explain the constant observation of only positive DCT erythrocytes in the above human erythrocyte cases of salmonellosis. Significantly, the above human erythrocyte abnormalities were detected at a time when severe anaemia, and sometimes haemoglobinuria, was observed. This indicates that the two processes may be causally related as they appear to be from the present studies on fowl typhoid.

There are other several gram-negative bacterial infections in man and animals in which increased haemolysis has been observed, and these include infections caused by E.coli (Horowitz, Javid and Spaet, 1960), Vibrio cholerae (De, Sengupta and Chanda, 1954) and Brucella (Bell and Irwin, 1938; Calder, Steen and Baker, 1939; Ferguson, Irwin and Beach, 1945). Overt haemolysis/

haemolysis has also been found associated with miscellaneous forms of gram-negative bacterial septicaemias (Brown et al., 1944; Brown, 1950; Kleeman and Epstein, 1957; Rapaport et al., 1964).

Apart from the demonstration of in-vivo erythrocyte sensitization, further evidence of erythrocyte abnormality associated with acute S.gallinarum infection is provided by the experimental results of alterations in osmotic fragility of the erythrocytes. The findings that the erythrocyte osmotic fragility is greatly increased and is reflected in a binodal curve, confirm the previous observations of Buxton (1960) and further demonstrate that this anaemia is of the haemolytic type. This conclusion is supported by the findings that increase in erythrocyte fragility has been noted in most cases of haemolytic anaemias (Dinning, 1962), and Suess et al. (1948) and Bolton (1949) have shown that in cases of haemolytic anaemia a characteristic binodal curve is always obtained. The above results also indicate that the abnormal fragility curves may be due to the sensitization of the erythrocytes rather than the presence of reticulocytes in the circulation, as has been suggested by Suess and colleagues (1948) to explain the binodal curves obtained in their studies. In support of this conclusion are the observations that the binodal curves in some of the infected chickens, especially in surviving animals, showed a temporary existence and also coincided with the appearance of the sensitized erythrocytes and not with the accompanying reticulocytosis. In addition, when control chickens were bled until they developed anaemia to the same degree as infected animals, and when reticulocytosis was nearly as severe, no binodal curves were obtained. Reticulocytes are relatively thin and show maximum lysis in weaker concentrations/

concentrations of salt than those required to lyse the majority of normal erythrocytes (Archer, 1965; Dacie and Lewis, 1968); that is, there is a 'shift to the left'. In the case of the experiments reported here, the 'shift was to the right', where a large proportion of the cells were haemolysed at a higher concentration of salt because of decreased resistance.

Significantly, the curves coincided with both the times at which sensitized erythrocytes were detected and when the haemolytic anaemia was observed. The development of these curves are therefore clearly characteristic of the generalised bacterial infection, and moreover, the coincidence of these abnormalities with the time of death suggests that all these phenomena may be causally, and intimately, related.

The decision to classify erythrocytes, which directly agglutinate with the Coombs antiglobulin serum (positive DCT) as separate Types III and IV sensitization, needs comment. Unlike Type III cells, the Type IV sensitized cells were first shown to agglutinate with the ICT, thus indicating that a bacterial antigen might have been adsorbed on to the erythrocyte surface. Their subsequent transfer to direct Coombs' positivity therefore indicated that these same cells have reacted with homologous antibody in-vivo. Thus, the strong possibility exists that the sensitizing factors of the Type IV erythrocytes are complexes of bacterial antigen and homologous antibody globulins. In contrast, the Type III cells, which may be due to bacterial antigen — antibody complex, could also equally be due to a number of other factors, either immunologically or non-immunologically acquired. The reasons for these conclusions are that these cells never agglutinated/

agglutinated when tested with the ICT, and direct Coombs' positivity simply indicated the in-vivo adsorption of globulins, and not necessarily antibody. Non-immunologic adherence of serum proteins to normal erythrocytes have been observed (Stratton and Jones, 1955; Warner, 1962) or reticulocytes (transferrin) Jandl, 1960; Sutherland, Eisentraut and McCall, 1963; Jensen, et al., 1965), and specific antibodies directed against endogeneous stromal antigens exposed by infectious agents (Dacie, 1963; 1968) may also give a positive result with the DCT.

Observations in the previous chapter had suggested a possible relationship between the duration of infection, the type of in-vivo erythrocyte sensitization, the severity of the haemolytic episode and mortality rate. To confirm this in a more general way, data from other aspects of these investigations are correlated. The results are presented as a histogram (Fig.21) which reveals a definite relation between the parameters enumerated above. Thus, animals dying very quickly after infection neither showed sensitization (Type I) nor any significant anaemia. In contrast, animals exhibiting the Type III sensitization were either the last to die or else survived. Moreover, the anaemia associated with this group was only moderately severe. Chicken exhibiting Type II sensitization invariably developed acute disease and died with severe anaemia. Similarly, this pattern was also observed in chicken with Type IV sensitization; that is, those in which a transition occurred from Type II to III. In general, the majority of chickens died between the fourth and sixth days after infection (73.6 per cent of total mortality) and during the peak period on the fifth day alone 33.8 per cent died. Fig.21 also shows that at this particular time all types of erythrocyte sensitization were found.

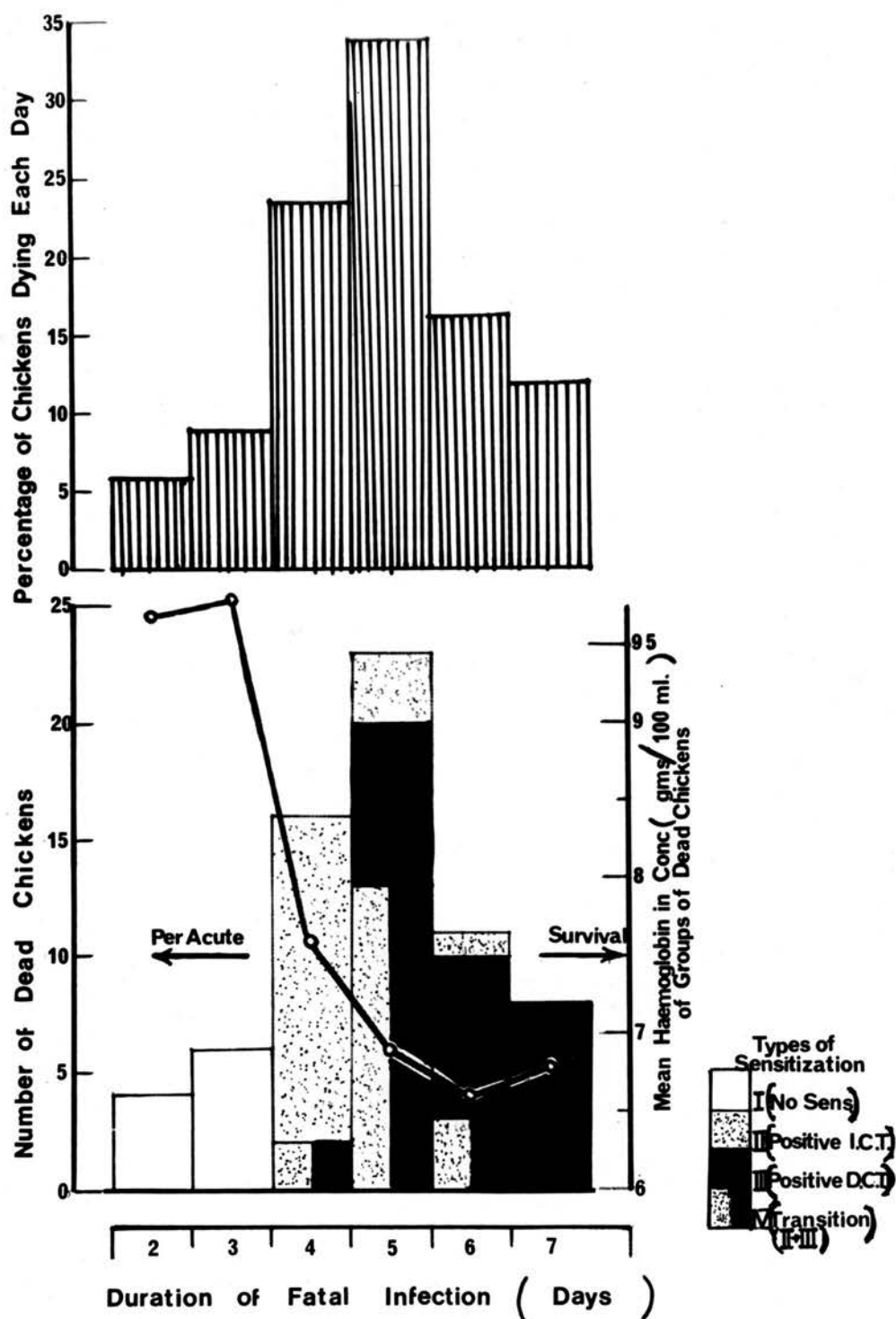


FIGURE 21. The Relationship Between the Type of in-vivo Erythrocyte Sensitization, the Severity of the Haemolytic Episode (Hb.conc.), the Rate of Mortality and Duration of Infection.

The significance of these relationships is not very clear. However, it is possible that in the per-acute cases, where chickens died without demonstrable sensitization or anaemia, death may have been due to abnormal susceptibility to endotoxin; as a result, death occurred before sufficient endotoxin was available to give rise to sensitization. As an explanation of Type II deaths it may be that here, combination with antibody also occurred but extremely rapid elimination of erythrocytes which occurred in these cases may have led to the failure to demonstrate it.

The continued persistence of Type III sensitized erythrocytes (that is, those detectable by DCT) may be due to the possibility that initially, the sensitized cells are rapidly removed from the circulation by the sessile or stationary macrophages of the RES and destroyed, but later, as the RES becomes increasingly impaired functionally, the sessile macrophages are unable to phagocytose and destroy all the sensitized cells presented to them. The excess cells therefore remain in the peripheral circulation where they may be detected continually. Peripheral erythrophagocytosis by monocytic cells (Zinkham and Diamond, 1952; Conway, 1953 and Jandl and Tomlinson, 1958) probably destroys some of these cells but this is unlikely to have any immediate impact on the overall numbers of sensitized cells for some time because of continuous erythrocyte sensitization and entry into the general circulation.

Alternatively, persistence may indicate a protective action by the coating globulin, as have been suggested for certain conditions of positivity of Coombs antiglobulin serum (Jandl and Simmons, 1957; Lindsey, Donaldson and Woodruff, 1966). It seems worthy of consideration that the acquisition of a coating of globulin/

globulin, though possibly disadvantageous from an immunological point of view, might also protect the erythrocytes from further destruction by the RES under certain conditions. The suggestion that a coating of globulin may be protective in various contexts is by no means new; the concepts in this regard have recently been reviewed by Thomas (1964). In the present context, it is suggested that a different type of antibody — protective rather than destructive — occasionally becomes adsorbed on the erythrocytes. Significantly, all the surviving chickens exhibited Type III erythrocyte sensitization.

An intriguing feature of the present experimental results is the apparent relation between the type of in-vivo erythrocyte sensitization and the time of appearance of complete haemagglutinating antibodies to S.gallinarum O-antigen during infection. Inspection of the individual erythrocyte sensitization patterns in the infected chickens (Figs. 10 - 16) reveals that animals in which complete haemagglutinins were produced exhibited the 3 positive types of sensitization of their erythrocytes. In contrast, in chicken showing no haemagglutinating antibody erythrocyte sensitization was never demonstrated. In general, however, early appearance of complete haemagglutinins appeared to relate to either Type III or IV sensitization, whereas late appearance of this type of antibody resulted in the formation of mainly Type II sensitized cells. These observations provide further evidence in support of the suggestion that an immunologically-mediated mechanism may be responsible for the development of the anaemia associated with this infection.

This study has also demonstrated that in acute S.gallinarum/

S.gallinarum infections in chickens, antibodies may be detected in the serum by the second day, using the antiglobulin haemagglutination test, and only later by the haemagglutination test. Similar observations have been made by Buxton and Davies (1963), but the antibodies were not characterized. Characterization of the immune response at the critical stage of the induction of the anaemia in these studies showed that a large amount of the specific antibody is present in the IgM fraction (Fig.20a, b). In addition, when daily serum samples from infected chickens were characterized, it was observed that a greater part of the early specific antibodies resided in the IgM fraction; the IgG antibodies appeared only in later serum samples. This sequential production of IgM and IgG is similar to that reported by Turner, Jenkin and Rowley (1963) from experiments with S.typhi-murium in mice, and by Allan, Duffus and Higgins (1968) and Duffus and Allan (1968) with chickens orally infected with S.gallinarum.

Considering that IgM is more proficient than IgG in agglutinating sensitized erythrocytes (Greenbury, Moore and Nunn, 1963; Grey, 1964; Benedict, 1965; Lindquist and Bauer, 1966; Burton and Mollison, 1968), and the finding that large amounts of specific IgM could be demonstrated during the haemolytic period, it is suggested that the antibody which may be directly involved in the immune rapid clearance and destruction of sensitized erythrocytes is of IgM class.

A striking feature of the experiments reported here needs comment. This was the persistence, although transitory, of positive ICT (Type II sensitized) erythrocytes in the presence of circulating anti- S.gallinarum (polysaccharide) antibody which would normally be expected to have reacted readily with the sensitized/

sensitized erythrocytes (Table 6; see also chicken nos. 2D, 4D and 11D of Figs. 10, 11 and 15 respectively), provided that the in-vivo sensitizing antigen is S.gallinarum polysaccharide. However, these cells were eventually cleared, presumably by the appearance of specific or high-affinity antibodies. Several explanations seem to be possible for the apparent paradox of Type II sensitized cells and, apparently, homologous antibody co-existing simultaneously in the circulation. The condition can be produced by heterologous antibody response to the adsorbed erythrocyte bacterial antigen, with varying binding capacities (Eisen and Siskind, 1964). An early low-affinity homologous antibody, but later, with the probable appearance of high-affinity specific antibody, such a reaction would readily occur, with consequent rapid clearance of the cells from the circulation as the present studies indicate. Similar patterns of reactivity and clearance will also be observed if, during this period, there is an absence of specific antibody reactive with the adsorbed antigenic determinant on the erythrocyte surface. Thus, the possibility of a different adsorbed antigen must also be considered. In-vivo alteration of adsorbed antigenic determinants may also render them unreactive to homologous antibody. This would, however, not seem to be the explanation of this condition since subsequent reaction of the sensitized erythrocytes with antibody, and rapid elimination of the same cells have been observed soon afterwards in chickens which eventually became very anaemic. However, as with the antigens of C.burnetti (Bakemeier, 1965) and penicillin (Epp, 1962), there may be a difference between the antibodies detected by cells to which S.gallinarum polysaccharide is/

is merely adsorbed in-vitro, and those to which the polysaccharide is firmly linked in-vivo, probably by a different chemical bond. It is also likely that continuous re-seeding of the circulation with sensitized erythrocytes, but non-comparable increase or change in specific antibody, would result in the persistence of these sensitized erythrocytes. This could conceivably happen if the process of sensitization of erythrocytes occurs somewhere, perhaps in the RES, from where the release of continuous stream of sensitized cells into the circulation is feasible. Whether this latter process represents an important cause of this condition remains to be determined.

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CHAPTER IV (PARTS I AND II)THE IN-VIVO ERYTHROCYTE SENSITIZING FACTOR : EVIDENCE FORA BACTERIAL LIPO-POLYSACCHARIDEGENERAL INTRODUCTION

The impression has been conveyed that in-vivo erythrocyte sensitization during bacterial infections is a rare phenomenon (Shumway et al., 1963; Dacie, 1967). However, in previous chapters it was indicated that in-vivo sensitization of erythrocytes occurs not infrequently in various bacterial diseases of man and animals.

The question immediately arose as to the nature of the sensitizing factor. The fact that lipo-polysaccharide can be easily adsorbed to erythrocyte in-vitro (Neter, 1956) suggests that this was the most likely bacterial component responsible for in-vivo sensitization. Although Buxton (1959a), Saito, et al. (1961), Young, et al. (1962), Springer and Horton (1964) and Springer (1964) have all suggested that bacterial polysaccharide antigens do adsorb on erythrocytes in-vivo during certain infections, unequivocal evidence to show this has not been put forward. The possibility that substances besides bacterial polysaccharides sensitize erythrocytes cannot be excluded, and evidence to clearly demonstrate the specific identity of the sensitizing factors is overdue.

The object of these studies was therefore to make detailed parallel studies between erythrocytes sensitized in-vitro to various degrees with S.gallinarum polysaccharide and in-vivo sensitized/

sensitized erythrocytes recovered from chickens with acute fowl typhoid. Comparative studies of this nature may enable more definite conclusions to be drawn as to the nature of the in-vivo sensitizing factor(s) and the degree to which in-vivo sensitized erythrocytes are specifically altered.

For ease of presentation, the data in this chapter has been divided into two parts :

- (1) Part I : Investigations based on the biologic and antigenic properties of the sensitizing factor(s). For example, the pyrogenic and leucocytic effects and specific antibody responses.
- (2) Part II : Evidence based on :
 - (a) Immunofluorescence.
 - (b) Attempts to elute and dissociate the sensitizing factors from the erythrocyte surface.
 - (c) Electrophoretic mobility studies.

PART I

INTRODUCTION

The possibility that the sensitizing factors might be a specific lipo-polysaccharide-endotoxin derived from the infecting salmonella organism — led to the use of procedures based on the known biologic and serologic properties of this material/

material as a means of possible identification of the in-vivo sensitizing components.

The pyrogenic effects of injected endotoxin, especially in rabbits, are well known (Thomas, 1954; Atkins, 1960; Keene, Silberman and Landy, 1961; Berlin and Wood, 1964; Kimball and Wolff, 1967), as well as its ability to induce characteristic changes in the leucocyte levels (Atkins and Snell, 1964; Ritts, Young and Arndt, 1964; Visscher, 1965; Sheagren, Wolff and Shulman, 1967). It was therefore considered that injection of washed, pyrogen-free suspensions of in-vivo sensitized erythrocytes, devoid of any contaminating bacterial organisms, would provide convincing evidence for the presence of an endotoxin-like factor, probably specific polysaccharide, on the erythrocyte surface.

It was also decided to determine if the sensitizing factor was immunogenic, and if so, to ascertain if the antibodies resulting from the injection of in-vivo sensitized cells reacted with extracted S.gallinarum lipo-polysaccharide.

MATERIALS AND METHODS

Animals: (a) Adult, 6-month old, female 'Hybrid 66' (Chunky Chick Ltd.) were fed, housed and infected with virulent S.gallinarum as previously described (Chapter II). Groups of 3 chickens were infected at intervals of 3 or 4 days in order to ensure a constant and regular supply of sensitized erythrocytes.

(b) Approximately 60 albino rabbits of both sexes, weighing between 2 and 2.5 Kgms. from the same colony, were used. Variability, especially in the pyrogenic response of individual rabbits, was kept to a minimum by observing precautions to prevent emotional/

emotional hypothermia (Grant, 1950). These included training in stocks, application of minimal restraint during the pyrogenic test, use of animals from a single source of supply, and standardizing the base-line temperature at 101°F to 102°F.

Collection and Preparation of in-vivo Sensitized Erythrocytes for

Injection: Erythrocyte samples from infected chickens were tested daily for sensitization by the ICT and DCT (Chapter II). Once sensitization had been observed, the animal was bled, and the red cells washed at least 5 times with pyrogen-free saline (BDH Ltd. Poole). The sterility of the erythrocyte sample was checked (vide infra) and a standard amount injected into normal rabbits for the estimation of the pyrogenic, leucocytic and antibody responses.

Erythrocyte Sterility Check: It was important that the inoculum of in-vivo sensitized erythrocytes should be absolutely free of any contaminating S.gallinarum organisms and any potential non-specific pyrogen. Consequently, sterility of the cell preparations had to be ensured before being injected. A special technique was developed to ensure this: the heparinized blood samples were first rendered completely free of leucocytes. The erythrocytes were allowed to sediment by gravity for between 1 and 2 hours at 37°C (Bradley and Oppenheim, 1967) and the supernatant leucocyte-rich plasma was removed. A deposit on erythrocytes was prepared by repeated washing and slow centrifugation and suspension after removal of remaining "buffy-coat". Then, narrow-bottomed Pasteur pipettes were used for the final preparation of purified erythrocyte inoculum. This was achieved by carefully breaking the bulbous bottom of the pipette with a pair of forceps after every centrifugation, /

centrifugation, and allowing the sedimented, packed erythrocytes to gradually drip into another pipette. The top white cells and a small upper portion of the red cells were discarded after each washing. This treatment ensured a relatively pure preparation of sensitized erythrocytes for rabbit inoculations. However, before such cells could be injected, the absence of any contaminating bacterial organisms was confirmed by microscopic examination of stained preparation of the cells or the inoculation of a loopful of the cell preparation into selective media, including Selenite broth, Brilliant Green agar and MacConkey lactose bile salt agar. After overnight incubation, samples from the fluid media were smeared and stained with either Leishman's or May-Grunwald stains, and examined microscopically.

Apart from cultured methods, purity and sterility were also investigated by direct staining of normal erythrocyte smears following their incubation with S.gallinarum organisms: serial dilutions of washed viable bacterial organisms were incubated with equal amounts of 1.5 per cent suspension of normal chicken erythrocytes for one hour. Samples from the highest and lowest dilutions, with some intermediate dilutions, were washed, smeared, stained and microscopically examined.

Thoroughly checking for sterility of the erythrocyte inocula necessitated the storage of the samples at 4°C overnight: the samples were used only when no growth was observed after examination of the selective and enrichment media.

Estimation of Minimal (and Maximal) Sensitizing and Pyrogenic

Doses: /

Estimation of Minimal (and Maximal) Sensitizing and Pyrogenic

Doses: The purpose of these preliminary experiments was, firstly, to find the approximate concentration of specific polysaccharide extracted from S.gallinarum which, when adsorbed on erythrocytes, would give the same range of antiglobulin haemagglutination titres as could be obtained with in-vivo sensitized erythrocytes taken from infected chickens; secondly, this procedure, by enabling cells to be sensitized in-vitro to the same extent as cells obtained from infected chickens, would permit direct comparison of the leucocyte and pyrogenicity tests, in rabbits, of in-vitro and in-vivo sensitized cells to be made.

A unit of pyrogenicity, based on the second fever response at the 3-hour point, has been introduced as the Minimal Pyrogenic Dose-3 hour (MPD-3) by Watson and Kim (1963). They defined this unit (MPD-3) as the amount of endotoxin required to produce 1°F rise in the second fever peak; this was estimated to be 0.004 µgm per Kgm. body weight. They also demonstrated that 100 MPD-3 in a rabbit, weighing between 1 and 2 Kgms., increased both the fever response to the upper limits of the linear dose-response curve (4°F increase) and rendered the contour of the curve more biphasic.

5 mls. of 1 per cent suspension of erythrocytes were sensitized in-vitro with 0.1 ml. of various concentrations of specific bacterial polysaccharide and titrated against doubling dilutions of pre-diluted homologous antiserum. (Unless otherwise stated, specific homologous antiserum used in these experiments refers to immune serum obtained from chronically infected chickens.)

The/

The results, summarized in Table 9 show that the 0.1 ml. of 2 to 3 μ gm per ml. of polysaccharide (endotoxin) would sensitize 5 mls. of 1 per cent suspension of chicken erythrocytes to give the same ICT titre as the average in-vivo sensitized erythrocytes from infected chickens (Chapter III). Thus, 0.5 μ gm. of salmonella polysaccharide will sensitize 0.1 ml. of packed, normal chicken erythrocytes to give the same ICT titre as in-vivo Type II sensitized erythrocytes. (This quantity of polysaccharide will, henceforth, be referred to as the "minimal sensitizing dose"). The results also gave an estimate of the quantity of polysaccharide needed to "maximally" sensitize the erythrocytes in-vitro for complete haemagglutination.

Preliminary studies, in which 1 ml. volumes of various percentage suspensions of minimally sensitized erythrocytes were administered intravenously into groups of rabbits, showed that the 70 per cent saline suspension of cells would give the best reproducible biphasic fever contours (Fig.22(B)). The same amount of control non-sensitized erythrocytes, injected similarly, developed only low-grade monophasic pyrogenic elevations in temperatures (Fig.22(D)). These experiments enabled a standard dose of in-vivo sensitized erythrocytes to be calculated. Thus, 1 ml. of 70 per cent suspension of erythrocytes — either in-vitro or in-vivo sensitized — was injected into rabbits for pyrogenic and leucocytic examination.

Injections in Rabbits: Rabbits were injected with normal or sensitized erythrocytes intravenously into the marginal vein to investigate both the pyrogenic, leucocytic and antibody responses.

In addition to the above, erythrocytes were injected,
in/

TABLE 9

*STANDARDIZATION OF SPECIFIC POLYSACCHARIDE FOR IN-VITRO 'MINIMAL' SENSITIZATION

*(FOR PYROGENIC DOSE-BIPHASIC RESPONSE AND AH-I TEST)

CONCENTRATION OF POLYSACCHARIDE FOR MINIMAL SENSITIZATION (0.1 ml. for every 5 ml. of 1% erythrocyte suspension)	DILUTIONS OF SALM. GALLINARUM ANTISERUM												
	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$	$\frac{1}{10240}$	$\frac{1}{20480}$
100 ($\mu\text{g/ml}$)	4	4	4	4	3	2	+++	+++	++	++	+	-	-
50 ($\mu\text{g/ml}$)	4	4	3	2	1	+++	+++	++	++	+	±	-	-
25 ($\mu\text{g/ml}$)	1	+++	+++	+++	+++	++	++	+	+	-	-	-	-
10 ($\mu\text{g/ml}$)	+++	+++	+++	+++	++	++	++	+	-	-	-	-	-
5 ($\mu\text{g/ml}$)	+++	+++	+++	+++	++	+	+	±	-	-	-	-	-
2.5 ($\mu\text{g/ml}$)	+++	+++	++	++	+	+	±	-	-	-	-	-	-
2.0 ($\mu\text{g/ml}$)	+++	+++	++	++	+	+	-	-	-	-	-	-	-
1.25 ($\mu\text{g/ml}$)	+++	++	+	+	-	-	-	-	-	-	-	-	-
1.0 ($\mu\text{g/ml}$)	++	+	±	-	-	-	-	-	-	-	-	-	-
<div>4 = COMPLETE HAEMAGGLUTINATION 3 = MODERATE HAEMAGGLUTINATION 2 = SLIGHT (PARTIAL) HAEMAGGLUTINATION 1 = TRACE HAEMAGGLUTINATION</div> <div>+++ = COMPLETE ANTIGLOBULIN HAEMAGGLUTINATION ++ = MODERATE ANTIGLOBULIN HAEMAGGLUTINATION + = SLIGHT (PARTIAL) ANTIGLOBULIN HAEMAGGLUTINATION ± = TRACE ANTIGLOBULIN HAEMAGGLUTINATION</div>													

4 = COMPLETE HAEMAGGLUTINATION
 3 = MODERATE HAEMAGGLUTINATION
 2 = SLIGHT (PARTIAL) HAEMAGGLUTINATION
 1 = TRACE HAEMAGGLUTINATION
 +++ = COMPLETE ANTIGLOBULIN HAEMAGGLUTINATION
 ++ = MODERATE ANTIGLOBULIN HAEMAGGLUTINATION
 + = SLIGHT (PARTIAL) ANTIGLOBULIN HAEMAGGLUTINATION
 ± = TRACE ANTIGLOBULIN HAEMAGGLUTINATION

in complete Freund's adjuvant (CFA), into groups of normal rabbits to investigate their immunogenicity. 0.3 ml. of packed erythrocytes, mixed thoroughly in CFA, was injected into each popliteal lymph node by the method described by Newbould (1965). The same rabbit also received 0.4 ml. cells in CFA intramuscularly and 0.4 ml. cells in saline intravenously. This procedure was repeated with a number of rabbits, injecting either Types II and III sensitized erythrocytes, in vitro sensitized or normal erythrocytes. Rabbits were bled on appropriate days when peak immune responses were expected.

Sterile, pyrogen-free disposable needles and syringes were used throughout.

Recording of Pyrogenic Response: Temperatures were taken at 15 minute intervals with a clinical thermometer inserted deep into the rectum. On the day before, the rabbits had been trained for 6 to 8 hours in specially constructed wooden stocks with loose fitting collars. On the day of study, temperatures were monitored for at least 1 or 2 hours prior to injections to ensure a steady base-line. Animals were used only if the base-line temperatures were between 101°F and 102°F . Febrile responses were plotted on a standard arithmetic graph paper.

Collection of Rabbit Blood Samples: Blood specimens for the leucocytic and antibody assays were obtained in 1 to 2 ml. samples from the marginal ear vein. Animals were bled on the day before and on the day they were to be injected with the sensitized erythrocytes. Individual samples for leucocyte counting were collected in disposable Sequestrene tubes, whilst samples for antibody/

antibody assay were allowed to clot at room temperature, and the serum then separated, inactivated and stored at -20°C until required.

Estimation of Leucocytic Changes: Blood samples, collected at regular intervals of 10, 30 minutes 1, 6 and 24 hours after injection of 1 ml. volumes of 70 per cent suspension of sensitized erythrocytes, were estimated for changes in total leucocyte counts by the procedure described by Archer (1965). The improved Neubauer counting chamber was used, together with 1.5 per cent glacial acetic acid to which was added 1 per cent gentian violet.

Specific Antibody Assay: Antibody assay was performed on pooled stored sera. It included those sera obtained from rabbits used in the pyrogenicity studies (vide supra), and also from groups of animals in which the immune response was investigated independently by intravenous and intra-lymph node injections.

S.gallinarum haemagglutinating antibody was determined using the indirect haemagglutination test described previously (Chapter III), except that rabbit erythrocytes were sensitized maximally with the alkali-treated specific polysaccharide from S.gallinarum. The titre was expressed as the reciprocal of the final serum dilution giving grossly visible agglutination. In all, more than 2 dozen rabbits were used to study the immune response.

Haemagglutinin-Absorption Test: This test was used both to confirm the specificity of the rabbit haemagglutinins produced after the injection of the sensitized erythrocytes, and to investigate the nature and character of the 'factor' sensitizing the erythrocytes during/

during infection

The specificity of the induced haemagglutinins was determined by pre-incubation of equal amounts of the rabbit antiserum and a known concentration of specific polysaccharide. The mixture was then titrated against maximally sensitized rabbit erythrocytes. 0.2 ml. amounts of the antiserum and the polysaccharide were used for the absorption, and the time for incubation at 37°C did not exceed one hour.

To establish the specificity of the in-vivo Type II sensitized erythrocytes, a similar procedure as described above, was employed. However, packed Type II in-vivo sensitized erythrocytes were pre-incubated with a specific chicken anti-S.gallinarum polysaccharide serum of known titre. Absorptions were carried out by adding 0.3 ml. antiserum to an equal volume of washed, packed in-vivo sensitized erythrocytes and incubated at 37°C for one hour. The contents of the tubes were regularly mixed, and the absorbed antiserum was then tested for any residual antibody, using chicken erythrocytes maximally sensitized in-vitro with S.gallinarum polysaccharide. Other batches of these cells were also used for control absorption tests on the antiserum.

Preparation of Specific Chicken anti-polysaccharide Serum: Specific immune serum were prepared in adult Rhode Island Red hens against S.gallinarum lipopolysaccharide by a double inoculation schedule. Chickens were first injected with 10 mg. of polysaccharide intravenously and then left for 21 days, when the antibody titres had stabilised at 20 per cent of the primary maximal titre (Mulholland, Jackson and Landy, 1965). Then they were re-injected with the same dose of polysaccharide. The chickens were sampled daily/

daily after the second injection, and when a reasonable titre had been produced — usually between 4 and 5 days — they were bled out. Sera from these animals were pooled, heat inactivated, final haemagglutinin titre determined and stored until required.

Preparation of rabbit Specific Anti-Chicken Immunoglobulin Sera:

Specific anti-chicken immunoglobulin sera were prepared in rabbits by the intra-lymph node injection of antigen-antibody complexes prepared by immuno-electrophoresis (Goudie, Horne and Wilkinson, 1966). The antigen used was chicken gamma globulin antibody, prepared by injecting chicken with bovine serum albumin (BSA) and precipitating the resultant anti-BSA with BSA at the various stages of the immune response. The various stages of production of specific anti-chicken γ -globulin serum were as follows:

(a) Antibody Production in Chickens (Anti-BSA): Adult Rhode Island Red hens were injected intravenously with 40 mg. BSA (Dreesman, Larson, Pinckard, Groydon and Benedict, 1965) and bled 6 days and 10 days after injection. The animals were again bled after repeated intramuscular injections of 10 mg. BSA.

(b) Preparation of Antigen-Antibody Precipitates: BSA in 7 per cent NaCl was double diluted and an equal volume of chicken anti-BSA was added to each test sample. The tests were carried out separately on the sixth, the tenth and the prolonged immunisation serum samples. The equivalence point of the precipitate was determined visually at room temperature. Required quantities of precipitate were prepared for the intra-lymph node injection of rabbits at the range of equivalence point. The reaction was allowed to proceed for 4 hours, then precipitates were washed 6 times in 7 per cent NaCl and reconstituted to a small volume/

volume (one quarter to one eighth of original antigen-antibody mixture in physiological saline.

(c) Preparation of Rabbit Anti-chicken γ -globulins: Adult rabbits of both sexes were given 0.1 ml. mixtures of chicken anti-BSA-BSA precipitates in complete Freund's adjuvant (CFA) by the intra-popliteal lymph node injection (Newbould, 1965). A month later, further injections of the same antigen-antibody mixture were given, one intravenously and several by the intramuscular route. Blood was collected 10 days later.

(d) Preparation of Immunoglobulin Antigens: Normal chicken serum was electrophoresed in 1 per cent Difco Noble Agar in barbitone acetate buffer (Oxoid)(pH 8.6) ionic strength, 0.05 by the method described in the previous chapter. Troughs were cut after the run and rabbit anti-chicken γ -globulin, prepared above (c), was allowed to diffuse for 48 hours. Three lines were seen in the gamma region, and these were identified as γ_{G_1} , γ_{G_2} and γ_M on the basis of similar appearance to these immunoglobulins in other species. These precipitin arcs were carefully excised and treated according to the method described by Goudie and colleagues (Goudie et al., 1966).

(e) Preparation of Specific Rabbit Anti-chicken Immunoglobulin Sera: Washed immunoglobulin complexes were injected into rabbits by the intra-popliteal lymph node technique. The method was followed as described above for raising anti-chicken γ -globulins in rabbits. Different rabbits were injected with either γ_{G_1} , γ_{G_2} or γ_M bands. The specificity of the antisera was confirmed by immunoelectrophoresis.

Before being used, the anti-IgG and anti-IgM sera were/

were mixed in equal proportions, inactivated of complement, absorbed of natural haemagglutinins and standardized so that the final specific antiglobulin serum employed in the present experiments was, in fact, a mixture of anti-IgG and-IgM.

Antiglobulin haemagglutination-Inhibition Test (AH-IT): It was essential that this test be properly standardized, and validity of the results tested, before being used in the investigations. Therefore, preliminary experiments were carried out to estimate the sensitivity and specificity of the test. This consisted of dilutions of homologous antiserum, decreasing concentrations of specific polysaccharide, and normal chicken erythrocytes sensitized minimally in-vitro. The results of these experiments, depicted in Table 10, show that complete inhibition of the reaction could be achieved when 0.1 ml. amounts of specific polysaccharide — concentrations ranging between 5 and 7 μ gm per ml. — were pre-incubated with the standardized, fixed dilution of homologous antiserum. The mixtures were later titrated, with in-vitro minimally sensitized cells as the indicator-cell system.

In the actual test, pre-absorptions were carried out by mixing together equal volumes of 0.1 ml. of a fixed dilution of S.gallinarum antiserum and serial quantities of homologous antigen (polysaccharide). After incubation at 37°C for one hour, the contents of the tubes were tested for residual antiglobulin haemagglutinins by the indirect antiglobulin haemagglutination test. 1.5 per cent suspension of in-vivo Type II sensitized erythrocytes was employed as the indicator-cell system. A drop of 1:50 dilution of adsorbed rabbit anti-chicken whole serum was used for the final agglutination stage. (The same in-vivo Type II/

Type II sensitized erythrocytes used in the earlier Haemagglutinin-Absorption test (vide supra) were tested with the AH-IT).

The control experiments included in this test consisted of sterile saline instead of polysaccharide.

Specific Indirect and Direct Coombs' Test (ICT and DCT): The same techniques as described in Chapter II were used, except that the requisite reagents were specific and purified. Hence, the antiserum used for the ICT was a chicken anti-S.gallinarum serum and for the DCT, the antiglobulin was a rabbit specific anti-chicken immunoglobulin serum (mixture of 19S and 7S).

Similar tests were also performed with the crude reagents; namely S.gallinarum antiserum (homologous antiserum) and rabbit anti-chicken whole serum. In addition, permutations of these reagents — specific and crude — were employed in similar, but essentially confirmatory, tests.

RESULTS

Preliminary results from these experiments have shown that completely purified and sterile preparations of in-vivo sensitized erythrocytes — free from contaminating leucocytes and viable bacteria — could be obtained for injections.

It was also demonstrated by a simple in-vitro staining-microscopic test that whole, viable bacterial cells are not adsorbed by erythrocytes, at least in-vitro. Microscopic examination of stained preparations of sensitized erythrocytes from infected chickens also gave negative results.

(A) Biologic Activity of in-vivo Erythrocyte Sensitizing Factors:

The intravenous injection of rabbits with in-vitro sensitized/

TABLE 10

STANDARDIZATION OF ANTIGLOBULIN-HAEMAGGLUTINATION-INHIBITION TEST *(WITH IN-VITRO MINIMALLY SENS. RBCs)

CONCENTRATION OF POLYSACCHARIDE IN SALINE PRE-INCUBATED WITH HOMOLOGOUS ANTISERUM TO INHIBIT AH-IT (0.1ml USED)	DILUTIONS OF COMBINATIONS OF ANTIGEN-ANTIBODY MIXTURES										
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{512}$	$\frac{1}{1024}$	
100 ($\mu\text{g/ml}$)	-	-	-	-	-	-	-	-	-	-	-
10 ($\mu\text{g/ml}$)	-	-	-	-	-	-	-	-	-	-	-
7.5 ($\mu\text{g/ml}$)	-	-	-	-	-	-	-	-	-	-	-
⁺ 5.0 ($\mu\text{g/ml}$)	$\frac{1}{2}$	-	-	-	-	-	-	-	-	-	-
2.5 ($\mu\text{g/ml}$)	++	+	+	-	-	-	-	-	-	-	-
2.0 ($\mu\text{g/ml}$)	+++	++	++	+	-	-	-	-	-	-	-
1.0 ($\mu\text{g/ml}$)	+++	+++	++	++	+	-	-	-	-	-	-
0.5 ($\mu\text{g/ml}$)	+++	+++	+++	++	++	+	\pm	-	-	-	-
0.25 ($\mu\text{g/ml}$)	+++	+++	+++	++	++	+	\pm	-	-	-	-
0.1 ($\mu\text{g/ml}$)	+++	+++	+++	++	++	+	+	-	-	-	-
0.05 ($\mu\text{g/ml}$)	+++	+++	+++	+++	++	++	+	\pm	-	-	-
CONTROL: SALINE	+++	++	+++	+++	++	++	++	+	-	-	-

+ = DEGREE OF POSITIVE ANTIGLOBULIN HAEMAGGLUTINATION

* = MINIMUM AMOUNT FOR INHIBITION \equiv AMOUNT OF SENSITIZING POLYSACCHARIDE

erythrocytes, and those obtained from infected chickens, demonstrated their common ability to elicit the characteristic pyrogenic and leucocytic reactions of bacterial endotoxins. (Figs. 22 and 23, and Table 11).

(1) Pyrogenic Responses:

The pyrogenic activities in rabbits of in-vitro and in-vivo sensitized erythrocytes are depicted in Figs. 22 and 23.

(a) Responses Following i/v Injection of in-vitro Sensitized Erythrocytes: All rabbits injected with minimally and maximally in-vitro sensitized erythrocytes elicited the typical biphasic fever responses. Injection of minimally sensitized erythrocytes provoked biphasic fever peaks between 1 and 2 hours (during the first fever peak) and between 3 and 4 hours (during the second peak). These are illustrated in Fig. 22B. The contours did not differ markedly from the responses obtained with maximally sensitized erythrocytes (Fig. 22A). In addition, the mean increases in the first and second fever peaks were not any higher with maximally sensitized erythrocytes. These observations are in agreement with the earlier findings by Watson and Kim (1963) that an endotoxin dose of 100 MPD-3 (found to be about 1 μ gm. in 1 Kgm. rabbit) was usually on the upper end of the semi-log linear dose-response curve, and that above this dosage, the contour of the curve neither became any more biphasic nor showed any further temperature rise in the second peak.

In contrast, the injection of erythrocytes, minimally sensitized in-vitro and then incubated with homologous antibody — thus carrying an antigen-antibody complex — provoked only/

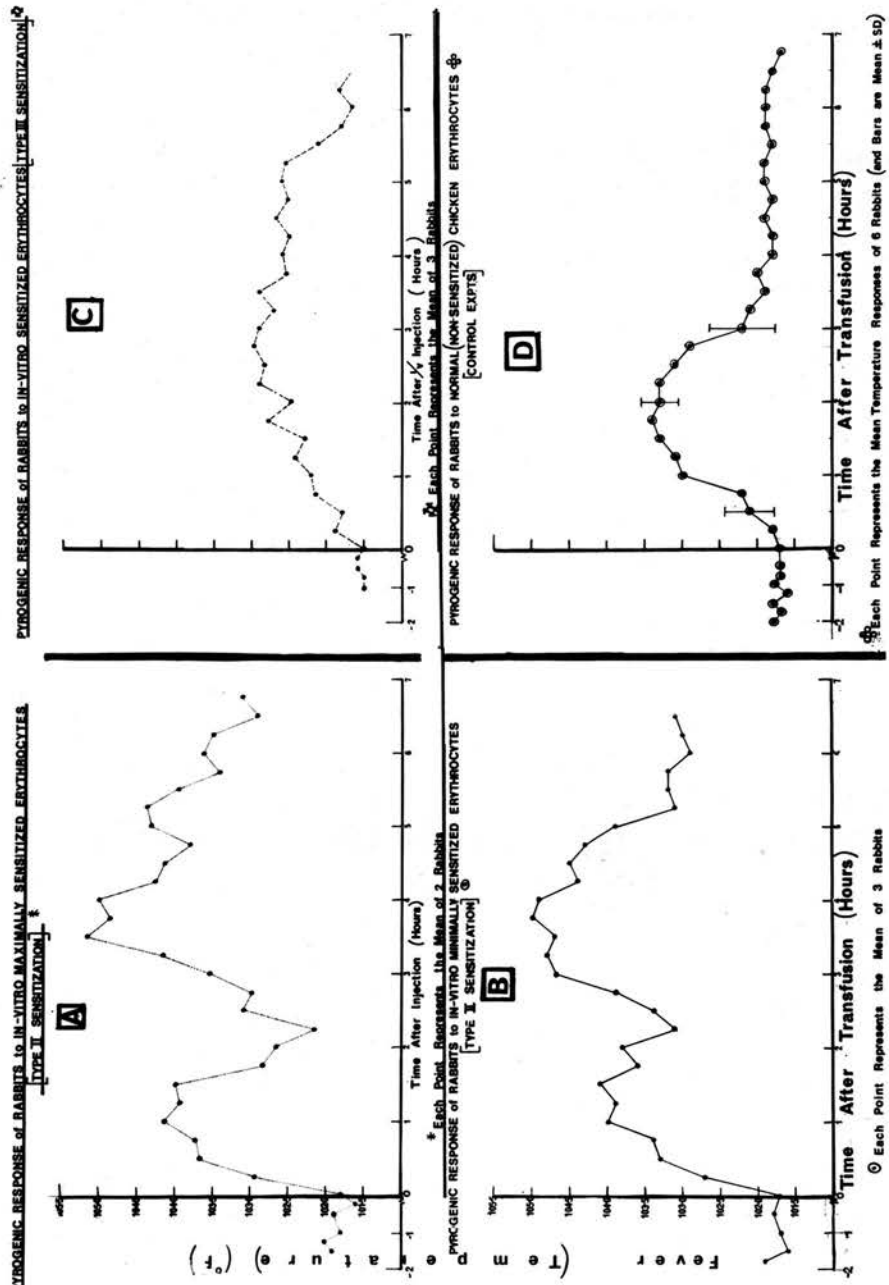


FIGURE 22. The Pyrogenic Responses of Rabbits Following i/v Injection of in-vitro Sensitized (and Non-Sensitized) Erythrocytes.

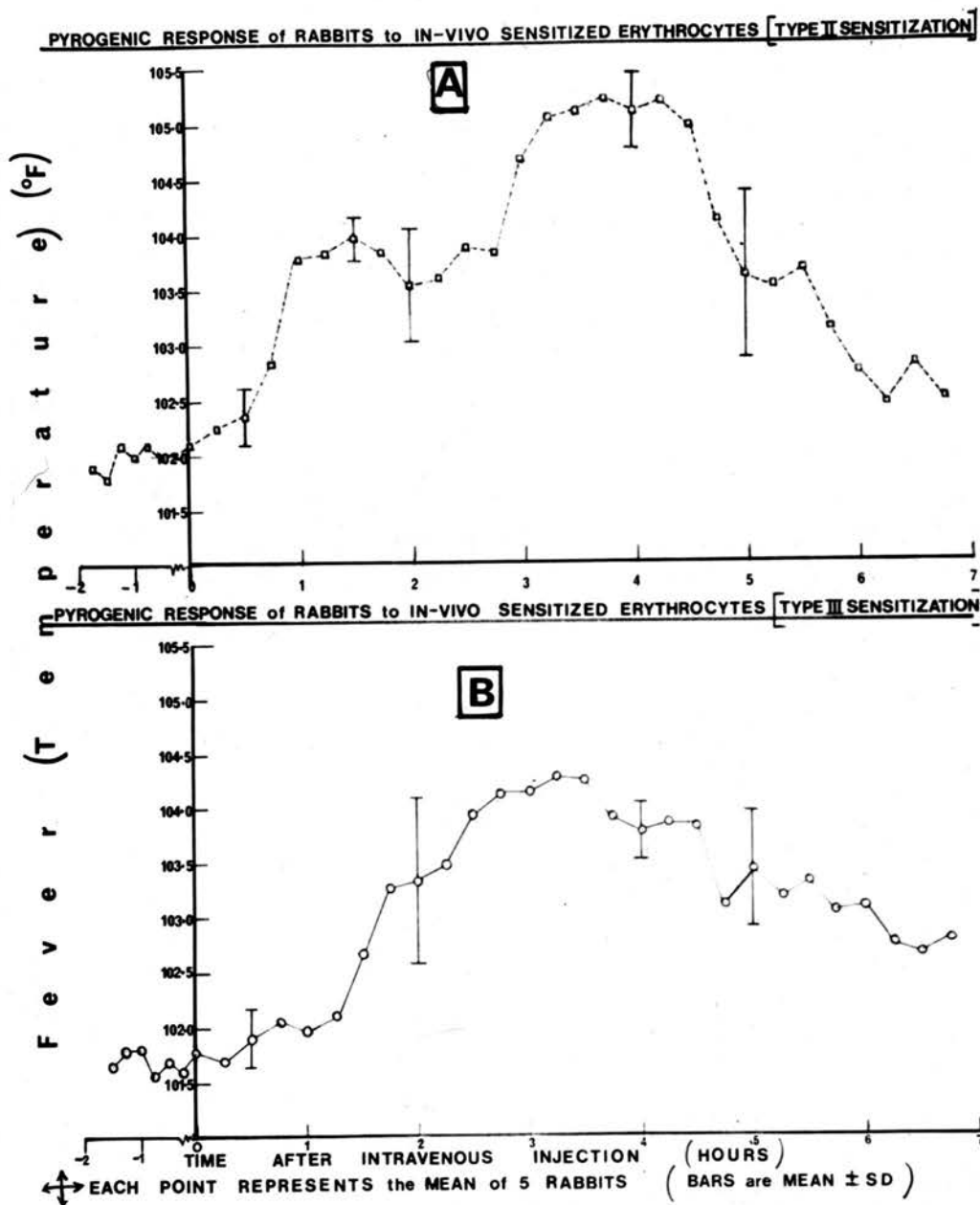


FIGURE 23. The Pyrogenic Responses in Rabbits Following i/v Injection of in-vivo Sensitized Erythrocytes.

only a monophasic response with a peak temperature rise of approximately 1.5°F , lasting several hours without remission, and ultimately returning to normal levels after more than 7 hours (Fig.22C). Similar findings were obtained with normal control rabbits injected with non-sensitized erythrocytes which subsequently developed low-grade monophasic elevations in temperatures of up to 1.5°F and lasting 1 to 2 hours (Fig.22D).

(b) Responses following i/v injection of *In-vivo* Sensitized Erythrocytes: The typical responses in rabbits after intravenous injection of *in-vivo* sensitized erythrocytes are depicted by Fig.23 (A and B). They demonstrate clearly the typical biphasic febrile responses characteristic of endotoxins. Rabbits injected with Type II sensitized cells developed average peak biphasic fevers of 2°F and 3°F at 1.5 and 4 hours respectively (Fig.23A). On the other hand, the fever curves tended to vary between biphasic and monophasic patterns in rabbits injected with *in-vivo* Type III sensitized cells (Fig.23B). In most cases, however, no distinct biphasic peaks of febrile responses were demonstrable, but the high temperatures of the monophasic response still persisted when monitoring was discontinued after more than 8 hours. In one or two cases in which rabbits developed distinct biphasic responses, the first peak took a comparatively longer time to develop (on average 2 hours), and this tended to shift the second peak to the right, which appeared usually after 4 hours. The overall mean fever increase with the Type III cells, however, was found to be approximately 2°F .

Comparison of the pyrogenic response patterns in rabbits/

rabbits injected with either in-vitro or in-vivo sensitized erythrocytes confirms the finding that the febrile responses in rabbits injected with in-vivo Type III sensitized erythrocytes are mostly of the monophasic type; in contrast, rabbits with in-vivo Type II sensitized cells elicited regularly biphasic responses.

(2) Leucocyte Changes:

The overall results of leucocytic changes in rabbits injected with chicken erythrocytes, variously sensitized, are summarized in Table 11. It shows that changes resembling those induced by endotoxins were demonstrated only in rabbits injected with in-vitro maximally sensitized erythrocytes. The characteristic initial, short, sharp 3-minute neutrophilia (Visscher, 1965) was however, not demonstrated with this type of cell. However, the subsequent decline to neutropaenic levels was shown to occur 30 minutes after injection of these particular cells. In general, however, the early reduction in leucocyte levels was small.

The leucocyte levels in some of the injected rabbits started to rise significantly above the normal values 6 hours after injection, but 24 hours later distinct leucocytosis was demonstrable in most of the rabbits injected with sensitized cells, particularly those injected with in-vitro sensitized and in-vivo Type II sensitized erythrocytes. The highest 24-hour-increase was obtained with the in-vitro maximally sensitized cells, which produced over 3-fold increase in leucocyte normal levels. There was also a 2-fold increase when both the in-vitro minimally, and the in-vivo Type II sensitized cells were administered; in contrast, in-vivo/

TABLE 11
LEUCOCYTE CHANGES IN RABBITS FOLLOWING INJECTION OF SENSITIZED ERYTHROCYTES

STATUS OF INJECTED CHICKEN ERYTHROCYTES	RABBIT NO. (PA)	LEUCOCYTE COUNTS ($\times 10^3/\text{mm}^3$)					
		MEAN OF PRE-INJECTION	10 MINS	30 MINS	1 HOUR	6 HOURS	24 HOURS
IN-VITRO 'MINIMALLY' SENSITIZED ERYTHROCYTES (TYPE II SENSITIZATION)	69	9.5	9.25	8.23	8.8	9.8	16.95
	83	10.0	8.5	6.9	8.45	10.55	19.25
IN-VITRO 'MAXIMALLY' SENSITIZED ERYTHROCYTES (TYPE II SENSITIZATION)	75	8.65	6.3	3.25	5.18	8.8	23.9
	56	9.85	5.9	4.1	5.0	8.1	20.5
IN-VIVO SENSITIZED ERYTHROCYTES (TYPE II SENSITIZATION)	107	8.65	7.75	6.85	7.45	9.5	14.8
	127	8.5	8.5	7.75	8.6	10.45	17.5
	133	8.23	8.5	6.35	7.5	9.55	18.0
	119	7.9	7.45	7.45	8.0	10.0	21.2
	124	9.2	8.3	6.95	7.2	9.45	15.95
	95	9.18	8.7	8.2	10.1	9.45	14.65
IN-VIVO SENSITIZED ERYTHROCYTES (TYPE III SENSITIZATION)	106	8.29	8.3	7.95	8.1	8.5	12.95
	88	8.15	7.8	7.9	7.3	7.8	10.5
	91	7.85	9.0	8.2	9.0	8.6	9.95
	146	9.0	8.6	8.5	7.95	8.0	10.4
	14	9.4	9.25	8.1	8.5	7.85	8.95
NORMAL, NON-SENSITIZED ERYTHROCYTES	20	9.0	8.9	9.2	8.9	9.0	9.2
	33	8.25	9.15	8.95	9.4	8.9	9.0
	38	7.2	8.65	8.3	8.23	9.1	8.75

in-vivo Type III sensitized cells either induced marginal increases or did not elicit any leucocytosis at all. Rabbits injected with normal, non-sensitized erythrocytes, and had responded with monophasic fever responses, did not show any changes in the leucocyte levels at any period after injections.

(B) Immunologic and Serologic Properties of Sensitized

Erythrocytes:

Erythrocytes, variously sensitized, induced high specific immune responses when injected into rabbits. The results of such experiments are summarized in Tables 12 and 13.

(1) Induction of Antibodies^{To} ~~In-vitro~~ and ~~In-vivo~~ Sensitized

Erythrocytes:

The antibody response of rabbits to a variety of inoculation schedules with in-vitro and in-vivo sensitized chicken erythrocytes, is shown in Table 12. There was consistent development of very high titres of specific haemagglutinins against in-vitro maximally sensitized erythrocytes. Moderate titres were also obtained when in-vitro minimally sensitized cells were administered. In contrast, no specific response to this antigen was detected in rabbits injected with non-sensitized (normal) erythrocytes. When in-vivo Type II sensitized erythrocytes were injected, high titres of haemagglutinins against adsorbed S.gallinarum polysaccharide were produced. In rabbits inoculated with in-vivo Type III sensitized red cells, however, the range of haemagglutinin titres was between 1:128 and 1:256, whereas titres of up to 1:8192 were obtained when in-vivo Type II sensitized cells were administered in complete Freund's adjuvant.

In/

TABLE 12

ANTIBODY LEVELS IN RABBITS FOLLOWING THE INJECTION OF SENSITIZED ERYTHROCYTES

STATUS OF INJECTED CHICKEN ERYTHROCYTES	ROUTE OF INJECTION	POOLED SERA ⁺		TIME OF SERUM COLLECTION (DAYS AFTER THE INJECTION ERYTHROCYTES)		INDIRECT HAEMAGGLUTININATING ANTIBODY TITRE*	
		NO. OF RABBITS	CLASSIFICATION			PRE-INJECTION	POST-INJECTION
NORMAL, NON-SENSITIZED ERYTHROCYTES	i/v	3	A	6	9	14	2
	i/in (CFA)						
	i/m (CFA)	2	B	21	28	-	0
IN-VITRO 'MINIMALLY' SENSITIZED ERYTHROCYTES (TYPE II SENSITIZATION)	i/v	3	C	7	10	14	2
							4096
IN-VITRO 'MINIMALLY' SENSITIZED ERYTHROCYTES (TYPE III SENSITIZATION)	i/v	3	D	9	11	15	0
							1024
IN-VITRO 'MAXIMALLY' SENSITIZED ERYTHROCYTES (TYPE II SENSITIZATION)	i/v	2	E	5	9	13	4
							65536
IN-VIVO SENSITIZED ERYTHROCYTES (TYPE II SENSITIZATION)	i/v	3	F	7	10	14	0
							1024
	i/in (CFA)						
	i/m (CFA)	2	G	21	30	-	2
	i/v						8192
IN-VIVO SENSITIZED ERYTHROCYTES (TYPE III SENSITIZATION)	i/v	2	H	7	12	-	0
							128
	i/in (CFA)						
	i/m (CFA)	3	I	21	28	33	0
	i/v						256

+ = TOTAL NUMBER OF INJECTED RABBITS FROM WHICH CLASSIFIED POOLED SERA WAS OBTAINED

* = HAEMAGGLUTININ TITRE EXPRESSED AS THE RECIPROCAL OF FINAL SERUM DILUTION

i/v = INTRAVENOUS INJECTION; i/in = INTRA-POPITALEAL LYMPH NODE INJECTION; i/m = INTRAMUSCULAR INJECTION

CFA = INJECTIONS MADE IN COMPLETE FREUND'S ADJUVANT.

In general, after intravenous injection of sensitized cells, a latent period of 2 to 3 days elapsed before any increase in anti-bacterial polysaccharide antibodies could be detected. It was also observed that much higher levels of specific antibodies were produced when Types II and III in-vivo sensitized cells were injected intramuscularly and by the popliteal lymph node route, using complete Freund's adjuvant, than when these cells were administered intravenously. However, with the former procedure, the latent period was approximately twice that following direct intravenous injection of erythrocytes.

(2) Specific Adsorption of Rabbit Haemagglutinins:

The specificity of the antibodies induced by the injection of sensitized erythrocytes described above was demonstrated by their complete absorption with homologous antigen (polysaccharide). The results of such experiments are depicted in Table 13, and show the complete disappearance of the antibody from the sera after incubation with S.gallinarum polysaccharide. With the exception of serum E (which was the highest titre induced by injection of in-vitro maximally sensitized cells), no antibody could be detected by the indirect haemagglutination test after pre-incubation with the polysaccharide.

These absorption tests have confirmed the specificity of the haemagglutinins induced by the injection of in-vivo sensitized cells; they also demonstrate conclusively that the erythrocyte sensitizing factor is a lipo-polysaccharide.

(3) Haemagglutinin Absorption from Specific Anti-polysaccharide Serum:

Further evidence for polysaccharide as the sensitizing/

TABLE 13

THE (SPECIFIC) ABSORPTION OF RABBIT HAEMA GLUTININS BY SALM. GALLINARUM POLYSACCHARIDE

CLASSIFICATION OF POOLED SERA (See Table 12.)	CONCENTRATIONS OF SALM. GALL. POLYSACCHARIDE USED FOR ABSORPTION ($\mu\text{g/ml}$) (0.2ml.)	*RABBIT HAEMAGGLUTININ TITRE	
		BEFORE ABSORPTION	AFTER ABSORPTION
C	2000	4096	0
D	1000	1024	0
E	5000	65536	4
F	1000	1024	0
G	2000	8192	0
H	1000	128	0
I	1000	256	0

* = RECIPROCAL OF FINAL SERUM DILUTION

sensitizing factor during infection was provided by the results of the haemagglutinin-absorption and antiglobulin haemagglutination-inhibition tests. Specific and purified reagents were used in these tests.

Washed, in-vivo sensitized Type II erythrocytes from 3 infected chickens and control, non-sensitized cells, were used to adsorb haemagglutinins from specific chicken anti-poly-saccharide serum of known titre. The results, recorded in Table 14, show that erythrocytes from all the infected animals absorbed only a proportion of the haemagglutinins from the antiserum. In contrast, control adsorption experiments, using the same quantity of in-vitro maximally sensitized erythrocytes, removed all demonstrable haemagglutinins from the antiserum.

These observations indicated that all the 3 erythrocyte samples from the infected chickens were either partially sensitized or that they consisted of a mixed population of cells with different degrees of sensitization — most probably, a heterogeneous mixture of non-sensitized (reticulocytes) and moderately or even maximally sensitized erythrocytes (Chapter VI). The observations also demonstrate that erythrocytes taken from the infected chicken are modified with a factor which specifically reacts with the anti-S.gallinarum O-antibody.

(4) Specific Inhibition of the Antiglobulin Haemagglutination Test:

The specificity and the approximate concentration of the sensitizing factor were again determined with an antiglobulin haemagglutination-inhibition test (AH-IT), whereby washed in-vivo Type II sensitized cells were used as the erythrocyte-indicator system. The results of such experiments, shown in Table 15, indicate/

TABLE 14

THE ABSORPTION OF HAEMAGGLUTINININS FROM CHICKEN ANTI-SALM. GALL. POLYSACCHARIDE SERUM BY TYPE II IN-VIVO SENSITIZED ERYTHROCYTES

SPECIFIC ANTISERUM ABSORBED WITH 0.3 ml. PACKED TYPE II SENSITIZED ERYTHROCYTES FROM:-	DILUTIONS OF SPECIFIC CHICKEN ANTI-POLYSACCHARIDE SERUM												
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{512}$	$\frac{1}{1024}$	$\frac{1}{2048}$	$\frac{1}{4096}$	$\frac{1}{8192}$
CHICKEN NO. 1 (ES)	+++	+++	+++	+++	++	+	±	-	-	-	-	-	-
CHICKEN NO. 2 (ES)	+++	+++	+++	+++	+++	++	++	+	±	-	-	-	-
CHICKEN NO. 3 (ES)	+++	+++	++	+	-	-	-	-	-	-	-	-	-
UNABSORBED ANTISERUM	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	±	-
0.3 ml. CONTROL (MAXIMALLY SENSITIZED ERYTHROCYTES)	-	-	-	-	-	-	-	-	-	-	-	-	-

+++ = COMPLETE HAEMAGGLUTINATION

++ = MODERATE HAEMAGGLUTINATION

+ = SLIGHT (PARTIAL) HAEMAGGLUTINATION

± = TRACE HAEMAGGLUTINATION

TABLE 15

ANTIGLOBULIN HAEMAGGLUTINATION-INHIBITION WITH TYPE II IN-VIVO SENSITIZED ERYTHROCYTES

CONCENTRATION OF POLYSACCHARIDE IN SALINE PRE-INCUBATED WITH HOMOLOGOUS ANTISERUM TO INHIBIT AH-IT (0.1ml USED)	TYPE II SENSITIZED ERYTHROCYTES FROM CHICKEN NO. (ES)																							
	1								2								3							
	DILUTIONS OF COMBINATIONS OF ANTIGEN-ANTIBODY MIXTURES																							
	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{512}$	$\frac{1}{1024}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{512}$	$\frac{1}{1024}$								
20 ($\mu\text{g/ml}$)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
10 ($\mu\text{g/ml}$)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
7.5 ($\mu\text{g/ml}$)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-								
5 ($\mu\text{g/ml}$)	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-								
2.5 ($\mu\text{g/ml}$)	++	++	+	-	-	-	-	+	-	-	-	-	-	-	+	-								
2.0 ($\mu\text{g/ml}$)	++	++	+	-	-	-	-	+	+	-	-	-	-	+	+	-								
1.0 ($\mu\text{g/ml}$)	+++	++	+	+	-	-	-	++	++	+	-	-	-	+	+	-								
0.5 ($\mu\text{g/ml}$)	+++	+++	++	+	+	-	-	+++	++	+	-	-	-	+	+	-								
- CONTROL: SALINE	+++	+++	++	++	+	-	-	+++	+++	++	+	-	-	+	+	-								

+ = DEGREE OF POSITIVE ANTIGLOBULIN HAEMAGGLUTINATION

* = MINIMUM AMOUNT OF POLYSACCHARIDE FOR INHIBITION = AMOUNT OF SENSITIZING LPS

indicate either minimal erythrocyte sensitization, or more probably, heterogeneity of the erythrocyte population taken from the infected animals. This was because only small amounts of specific polysaccharide were required to inhibit antiglobulin-haemagglutination with each of the cell samples.

Type II in-vivo sensitized cells from chicken number 3(ES) showed the highest sensitivity, and therefore the greatest degree of sensitization, since they could still detect antibodies in mixtures where concentrations in excess of 10 μ gm. per ml. polysaccharide had been used to pre-absorb the initial homologous antiserum. The minimum amount of specific polysaccharide which produced complete inhibition of the haemagglutination test was smallest with sensitized erythrocytes from chicken no. 2(ES) (5 μ gm. per ml.); and haemagglutination of erythrocytes from chicken no. 1(ES) was inhibited by only marginally higher concentration of polysaccharide(7.5 μ gm. per ml.)

(5) Serologic Tests with Specific Reagents using the ICT and DCT;

Using specific reagents (that is, chicken anti-S.gallinarum polysaccharide serum and rabbit anti-chicken immunoglobulin serum), the presence of polysaccharide and an antigen-antibody complex specifically adsorbed on erythrocyte surfaces, was unequivocally demonstrated (Table 16). The summarised results in this Table indicate that the sensitizing factor is a polysaccharide antigen derived from S.gallinarum.

All the Type II in-vivo sensitized erythrocyte samples from the 4 infected chickens produced varying titres with the combination of the specific reagents. These titres did not substantially alter when various permutations of reagents, crude (homologous/

(homologous antiserum and rabbit anti-chicken whole serum) and specific (anti-S.gallinarum polysaccharide serum and rabbit anti-chicken immunoglobulin serum), were used. For example, with erythrocytes from chicken number 9(ES) and employing crude reagents, essentially similar titres were recorded as when specific reagents were used (Table 16). Similarly, with Type III in-vivo sensitized cells, the titres were unchanged when examined using both crude and specific reagents.

TABLE 16

DETECTION OF TYPES II AND III SENSITIZED ERYTHROCYTES WITH SPECIFIC REAGENTS

NATURE AND SPECIFICITY OF ICT AND DCT REAGENTS	TYPE II SENSITIZED ERYTHROCYTES FROM CHICKEN NOS. (ES)				TYPE III SENSITIZED ERYTHROCYTES FROM CHICKEN NOS. (ES)			
	8	9	10	11	4	5	6	7
	*FINAL INDIRECT COOMBS TITRE				*FINAL DIRECT COOMBS TITRE			
'CRUDE'	HOMOLOGOUS ANTISERUM (HA) + RABBIT ANTI-CHICKEN WHOLE SERUM (RACWS)							
	256	128	512	64				
SPECIFIC	RACWS							
	128	128	256	32	1024	64	128	128
MIXED	SPECIFIC ANTI-POLYSACCHARIDE SERUM (SAPS) + SPECIFIC RABBIT ANTI-CHICKEN GAMMA GLOBULIN SERUM (SRACGS)							
	SRACGS							
	HA + SRACGS							
	128	256	512	64				
	SAPS + RACWS							
	256	128	256	32				

* = RECIPROCAL OF DILUTIONS OF HOMOLOGOUS ANTISERUM AND ANTIGLOBULIN SERUM

PART IIINTRODUCTION

Three further techniques were used to investigate the nature of the sensitizing factor(s) in order to provide more direct evidence than that already accumulated. These were:

(a) immunofluorescence; (b) elution and extraction procedures, and (c) changes in the electrophoretic mobilities of the in-vivo sensitized cells.

(a) Immunofluorescent technique: The usefulness of this technique for the study of tissue antigen-antibody systems is well recognised (Nairn, 1969). Although many problems are encountered when erythrocyte antigen-antibody systems are being investigated by this method, (Alexander, 1958; Whitaker, Zuelzer, Robinson and Evans, 1959; Cohen, Zuelzer and Evans, 1960; Lee and Vazquez, 1962) it has been shown that the commonly observed non-specific fluorescence when tissues are treated with fluorescent (conjugated) sera is not a problem with erythrocytes (Cohen et al., 1960).

A literature search showed that there have been no recorded studies so far on the use of this technique in demonstrating in-vivo erythrocyte surface changes during bacterial infections. It was thought that this technique could be utilized in demonstrating the sensitizing factor(s) in situ.

(b) Elution and Extraction Procedures: The presence of cells ^{to} positive/direct Coombs' test (Type III) cannot, on their own, be accepted as evidence that specific antibodies were being produced against an adsorbed polysaccharide, for a reaction between red cells and anti-red cell antibodies has to be excluded. There is also/

also the possibility that the in-vivo Type III erythrocyte sensitization may be due to non-specific adsorption of serum globulins other than antibodies (Stratton and Jones, 1955; Jandl, 1960; Warner, 1962; Sutherland, Eisentraut and McCall, 1963; Jensen, 1965). Attempts were therefore made to elute, dissociate and serologically identify the in-vivo Type III sensitizing factor(s).

All antigen-antibody reactions are probably reversible, and in those instances where the reaction is said to be irreversible, it is most likely that observations were not continued for sufficient time (Hughes-Jones, 1963). In dealing with an antigen-antibody mixture, only one of which was adsorbable on untreated normal erythrocytes, Lowenthal and Lamana (1953) utilised erythrocytes for the separation of botulinal haemagglutinin and its toxin. Isliker (1957) has found that lowering the pH to below 3.5 is a suitable method for dissociation of immune-complexes, because antibodies are stable under these conditions. In spite of the recent finding by Hjort (1968) that dissociation of immune-complexes under these conditions was incomplete, it was decided that after extraction of the complexes from the Type III sensitized cells, the eluate factors were to be dissociated under similar acidic conditions. With this approach the separation of the polysaccharide moiety of the eluate from the antibody portion could be effected with the normal erythrocyte acting as an adsorbing surface for the bacterial antigen.

(c) Electrophoretic Mobility techniques: It has been known for many years that red cells of various species move at a mobility unique/

unique for a particular species (Abramson, 1929) and disease also alters the electrophoretic mobility of the erythrocytes as a consequence of changes in the net cell surface charge (Brown, 1933; Redmond, 1948; Rottino and Angers, 1962; Domnert and Dimopoulos, 1966; 1967). The alteration of an erythrocyte surface, with special reference to in-vivo sensitization during infection, raised the possibility that a change in the electrophoretic mobility of such cells, as compared to normal erythrocytes, might be demonstrable. Consequently, an electrophoretic study of sensitized and non-sensitized chicken erythrocytes was undertaken to determine if such changes do occur. An apparatus was designed specially for this purpose.

MATERIALS AND METHODS

Fluorescent-Antibody (FA) Technique: The immunofluorescent technique used in these investigations was an adaptation of the various FA procedures as applied to red cell systems by the authors mentioned above. These authors had used many procedures to overcome the numerous problems encountered. The "quenching" phenomenon (Gitlin, et al., 1953) for instance, was overcome either by pre-treatment of the erythrocytes with chelating agents (e.g. Versene)(Whitaker, et al., 1959), or by enhancement of the number of fluorescent antibody molecules that can be attached to the red cells by means of the multiple-antibody layer method of labelling (Cohen et al., 1960). The "trapping" phenomenon, which occurs in experiments involving agglutinating antibodies (Cohen, 1960) was controlled by the use of either labelled normal serum/

serum in conjunction with unlabelled specific antiserum; or washed agglutinates were suspended in normal serum, rather than saline, prior to the addition of the conjugated sera.

Buffers: Three different buffer solutions were used in these experiments.

(a) Difco FA Phosphate buffer dried: This was a standardized phosphate buffer powder yielding, on dehydration, a buffer solution with a pH of 7.2. It was used for making all the dilutions and washings of erythrocytes and slides.

(b) Phosphate Buffered Saline (PBS): This had a pH of 7.2 and contained 0.01 M phosphate and 0.15 NaCl. It was prepared by dissolving 1.07 gms. Na_2HPO_4 , 0.39 gm. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 8.5 gms. NaCl in one litre of distilled water. This buffer was used for the conjugation of the antiserum and all dialysis work. It was also the buffer of choice as the eluent buffer in Sephadex G25 fraction of the dialysed conjugated antiserum.

(c) Borate Buffer: This had a pH of 9.0 and a molarity of 0.25, and was prepared by mixing together volumes of iso-molar boric acid and sodium hydroxide (NaOH): 100 mls. of 0.5M boric acid and 72.6 mls. of 0.5M NaOH.

Sensitized Erythrocytes: The techniques for infection of chicken and subsequent recovery of in-vivo sensitized erythrocytes, and the methods of in-vitro minimal and maximal erythrocyte sensitization, have all been previously described (Chapters II and IV, Part I).

Preparation of Antisera: The preparation of specific chicken anti-S.gallinarum polysaccharide serum, and the rabbit anti-chicken whole/

whole serum, were as described previously (Chapters III and II respectively).

Conjugation of Rabbit Anti-Chicken γ globulin Serum : A

modification of the method described by Lee and Davidsohn (1965) was used. The principle of this method was the conjugation of anti-whole serum, instead of anti-globulin serum, with fluorescein iso-thiocyanate (FITC) and the precipitation of the conjugated globulins with half-saturated ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$. In this way, not only albumin, but also most of the free fluorescein is removed with the supernatant. After this only one dialysis, with 3 changes of buffer, is normally required. Although this procedure generally makes the use of chromatography unnecessary, it was decided to obtain a pure product by fractionating the conjugate with Sephadex G25.

Procedure for Conjugation: 20 mgms. of FITC were sprinkled on filter paper at the bottom of a beaker, and a little acetone poured on. The FITC spreads to cover the whole surface of the filter paper as a result; then 15.25 mls. of PBS and 2.25 mls. of the borate buffer were added and mixed with a magnetic stirrer. 5 mls. of the rabbit anti-chicken whole serum (RACWS) were added to the mixture while being stirred; the stirring was continued for another 2 to 3 hours, then the mixture was left overnight at 4°C . Linkage of FITC with globulins takes place spontaneously at alkaline pH 8.5 to 9.0 by covalent linkage.

The next day, the conjugated globulin fraction of the antiserum was obtained by the addition of saturated $(\text{NH}_4)_2\text{SO}_4$ to the point of half-saturation. This was done by adding an equal volume/

volume (22.5 mls.) of saturated $(\text{NH}_4)_2\text{SO}_4$ slowly to the conjugated antiserum in buffer solution and stirring for 30 minutes. The mixtures were then left at room temperature (20°C) for 2 hours and the supernatant solution removed by centrifugation at 8000 r.p.m. for 20 minutes. The precipitate was then washed twice with half-saturated $(\text{NH}_4)_2\text{SO}_4$ (50 per cent) and either suspended in a minimum of distilled water or dissolved in 5 mls. or less of PBS. The $(\text{NH}_4)_2\text{SO}_4$ was then removed by dialysis overnight at 4°C against at least a litre of PBS, with continuous agitation and mixing. Three changes of buffer were normally required to remove all the free $(\text{NH}_4)_2\text{SO}_4$ and fluorescein.

The conjugated protein solution was then put on top of Sephadex G25 column and washed through with PBS. This rapidly restored the conjugate-mixture to neutral pH and isotonicity, and at the same time, removed the free and loosely bound FITC. Collection of conjugated samples from the column started when the first yellow band approached the bottom of the column. The contents of the tubes were pooled and liver-powder was added to the pooled fraction and left overnight. This procedure removed all activity against non-specific proteins. The conjugated globulin preparation was then filtered through a sterile Seitz filter, and the supernatant was removed and stored at -20°C .

Samples of the final conjugated globulins were tested for specificity by immuno-electrophoresis against normal chicken whole serum. In addition, the conjugated antiserum was thoroughly absorbed of any natural haemagglutinins with normal chicken erythrocytes before use.

Experimental/

Experimental Design for FA Investigations:

Preparation of Erythrocytes: The method of erythrocyte staining and mounting was modified from the techniques described by Janković (1959) and Lee and Vasquez (1962).

(i) Fluorescent Staining Methods: The indirect staining method was used with the Type II in-vivo sensitized cells, whilst, the Type III sensitized cells were labelled by the direct staining method.

Both the two-and three-layer indirect labelling techniques were used.

Two Layer Method: Equal volumes (0.5 ml.) of 1.5 per cent suspension of Type II cells and specific, unconjugated specific anti-polysaccharide serum (CAPS) were added to each other and incubated at 37°C for 1.5 hours. The mixture was shaken and mixed thoroughly at regular intervals during incubation; at the end of incubation time the erythrocytes were washed 3 times in Difco FA phosphate buffer. Preliminary experiments were performed to determine the optimal dilution of the absorbed conjugated rabbit anti-chicken γ -globulin serum (conjugated RACGS) which had been previously inactivated of complement activity. Different dilutions of the conjugated RACGS were titrated against single drops of the 1.5 per cent washed sensitized erythrocyte suspension. Samples were examined after incubation, washing and mounting for the clearest and most satisfactory fluorescence. This was found to be 1:2 dilution of the prepared conjugated RACGS.

One drop of the 1.5 per cent suspension of the sensitized/

sensitized cells was incubated with 5 drops of the conjugated RACGS for one hour at 37°C. Afterwards, the reaction mixture was washed 4 times with PBS with vigorous shaking mixing. Samples from the packed cells, suspended in mounting fluid (vide infra) were examined microscopically.

Three Layer Method: The same procedure as above was followed, but 5 drops of unconjugated RACGS were added instead. The mixtures were incubated and washed as described above. Afterwards, 5 drops of decomplexed Difco conjugated goat anti-rabbit γ -globulin serum were added to the washed cells, incubated, washed, mounted and examined.

Type III in-vivo sensitized cells were, however, prepared for examination by the direct staining method, utilising either the single-or the double -layer techniques.

The Single-layer direct staining procedure: This consisted of incubation of one drop of 1.5 per cent erythrocyte suspension in PBS with 5 drops of undiluted conjugated RACGS for one hour at 37°C. The cells were then washed, mounted and examined.

The Double-layer direct method: The prepared test cells were pre-incubated in conjugated RACGS, washed and then subjected to labelling with undiluted Difco conjugated goat anti-rabbit γ -globulin serum.

Control Experiments: Serological specificity of immunofluorescence in these experiments was demonstrated by control experiments involving in-vitro, in-vivo and non-sensitized erythrocytes. Blocking experiments with conjugated and unconjugated specific antisera were also performed.

Using/

Using either the Two- or Three-Layer techniques where appropriate, the following control experiments were undertaken:

- (a) Studies with in-vitro maximally and minimally sensitized erythrocytes.
- (b) Normal erythrocytes subjected to labelling with either of the conjugated antiserum.
- (c) In-vivo sensitized cells exposed to conjugated RACGS which had previous been absorbed with specific unconjugated chicken γ -globulin serum.
- (d) The same in-vivo sensitized cells were pre-treated with unconjugated RACGS before treatment with conjugated RACGS.
- (e) Type II sensitized cells were also subjected to conjugated RACGS without pre-incubation in specific chicken anti-polysaccharide serum.

(ii) Mounting of Erythrocyte Preparations: One or two drops of Difco FA mounting fluid, which consists of 50 per cent buffered saline-glycerine, (pH 7.2), were added to the final packed labelled cells. Using a Pasteur pipette, the ^{agglutinated} mixture was thoroughly broken up ~~of agglutinates~~ to produce an almost homogeneous suspension. A small drop of this wet preparation, in mounting fluid, was then transferred on to special Chance Glass slides (1.03 mm. thick) and gently flattened with coverslips (22 x 40 mm.) cut from Chance Glass.

Mounts were made relatively permanent by sealing with nail varnish, and examined microscopically after the wet mounts had 'settled' under the fluorescent microscope.

(iii) Examination of Wet Mounts: From the technical point of view/

view the specific fluorescence was achieved by examining wet preparations with alternatively dark-field illumination and with ultra-violet light, and a 40 x objective. (The failure of other investigations with the FA method as applied to erythrocyte systems may be attributable, at least in part, to the examination of dried preparations (Cohen et al., 1960).

(iv) Photomicrography: A Zeiss fluorescent microscope, with an attachment camera and an Ilford HP 4 (Black and White) film, was used in photographing the wet preparations. The average exposure time was 20 minutes.

Elution, Dissociation and Serology of Sensitizing Factor(s): A quantity of Type III in-vivo sensitized erythrocytes from infected chickens was checked for sterility (Chapter IV, Part I), and the sensitizing factor(s) was extracted by combining and modifying the methods previously used for preparing erythrocyte stroma, (Kominos and Rosenthal, 1953) and eluate (Landsteiner and Miller, 1925). As a rule, elutions were carried out on the same day that sterility of the cells was confirmed.

Preparation of Erythrocyte Stroma: Packed, sensitized cells were washed 6 times with copious amounts of normal saline, and after being firmly packed by centrifugation, were haemolysed with 10 volumes of pyrogen-free distilled water. Then, by dropwise addition of N.HCl until the pH was in the range of 5.6 and 5.8, precipitation of the stroma was readily carried out. The precipitated stroma was then washed repeatedly with phosphate buffered pyrogen-free distilled water (pH 5.6 - 5.8), until almost white or just slightly brown tinged, when the elimination of haemoglobin was almost complete. After firm packing, it was then ready/

ready for elution.

Preparation of Eluate from Stroma: The well-packed stroma was suspended in twice, equal or half its volume of normal saline — depending on the desired final concentration of eluate factors — and thoroughly mixed. It was incubated at 56°C for 15 minutes and then centrifuged at high speed in pre-warmed tubes by putting water at 56°C in the centrifuge buckets. The supernatant was collected and stored at -20°C until needed.

Dissociation of Eluate Factors: The elution of the sensitizing factors from the cell surface would in itself be of little value were it not possible to dissociate and demonstrate the actions of the constituent parts contained in the eluate.

Dissociation of the eluate was carried out by lowering the pH to below 3.5 (Isliker, 1957) and the released antibody in the eluate was then titrated with in-vitro maximally sensitized erythrocytes after the eluate had been pre-adsorbed with normal chicken erythrocytes. The latter cells were also later titrated against serially diluted specific antiserum (vide infra).

Antibody which has been exposed to acid will retain its serological activity even though its physico-chemical properties may be markedly altered (Isliker, 1964). Similarly, acid hydrolysis of lipo-polysaccharide does not affect its erythrocyte sensitizing properties (Neter, Gorzynski and Westphal, 1955; Neter, Lüderitz, Gorzynski and Eichenberger, 1956; Tsumita and Ohashi, 1964; Springer et al., 1966).

Demonstration of the Actions of Eluate Constituents: The dissociated eluate was divided into 2 parts; one part for the experimental/

experimental test, and the other portion was used for the control test.

(i) Serological test for the Presence of a Specific Polysaccharide: Normal packed chicken erythrocytes and half of the total volume of the prepared eluate were mixed in a ratio of 1 in 50 respectively. The mixture was then incubated for one hour, with periodic gentle mixing. After centrifugation the supernatant was carefully removed and stored. The packed cells, duly treated with the undiluted eluate factor(s), were washed 3 times in normal saline and a suspension of 1 per cent of this made in saline. 0.2 ml. volumes of the suspension were added to doubling serial dilutions of S.gallinarum antiserum, using rabbit anti-chicken γ -globulin serum in an indirect antiglobulin haemagglutination test (Chapter III).

(ii) Serological test for the Presence of Specific Antibody: The stored supernatant from above was reabsorbed again with a larger amount of packed normal erythrocytes. After centrifugation and packing, the supernatant was removed and serially diluted. The titre of the factor in the eluate was determined, using a suspension of in-vitro maximally sensitized erythrocytes and specific antiglobulin serum.

Control experiments to determine the specificity of above reactions consisted of utilization of the other half of the eluate, as mentioned above. This was incubated with normal erythrocytes, and the supernatant stored. The treated-cells were washed and 1 per cent suspension of this tested against specific antiglobulin in a direct Coombs' test (Chapter II).

The other control experiments consisted of absorption/

absorption of the stored supernatant from above for a second time with normal erythrocytes. Serial dilutions of this supernatant solution were titrated against a suspension of normal erythrocytes, using single drops of the standardized specific antiglobulin in an ICT.

Further control experiments performed consisted of determination of activity against normal chicken erythrocytes by:

- (a) specific S.gallinarum antiserum
- (b) specific rabbit anti-chicken γ -globulin serum.
- (c) sera from chickens which had previously exhibited Type III in-vivo sensitization during the course of these experiments.

In addition, the in-vivo Type III sensitized cells — a portion of which an eluate had previously been obtained — were tested for specificity, using a specific anti-chicken γ -globulin serum.

Measurement of Electrophoretic Mobility:

Determination of the surface charge density of erythrocytes may be made using an electrophoretic technique to measure the electrophoretic mobility (Bangham, Flemans, Heard and Seaman, 1958; Hunter, 1960). This method, although not well known or widely used, may be employed to obtain an index of chemical changes on the erythrocyte surface (Hanig, 1948; Bateman and Zellner, 1956; Bateman, Zellner, Davis and McCaffrey, 1956; Hopps and Dengles, 1957; Davies, 1958; Cook, Heard and Seaman, 1961; Seaman and Uhlenbruck, 1962; Agarwal and Hjertén, 1964).

The purpose of the present experiments was to establish/

establish a satisfactory, simple and acceptable method for studying erythrocyte electrophoretic mobility changes which could be used as an index of in-vivo erythrocyte sensitization. The present technique was therefore designed to ensure this.

(a) The Buffer System: The buffer solution was prepared using the method reported by Schideler (1960). It contained: 750 mls. of 10 per cent sucrose solution; 151.5 mls. of M/15 disodium phosphate solution, and 36 mls. of M/15 potassium dihydrogen phosphate solution. The alkaline sodium phosphate and the acidic potassium phosphate solutions were mixed and sterilised by filtration. The sucrose solution was prepared daily, and 187.5 ml. of the sodium-potassium phosphate mixture was added to prepare a final 10 per cent sucrose-phosphate buffer solution with a pH of 7.4 and ionic strength of 0.035.

(b) The Electrophoresis Apparatus: The simple apparatus used in this study was constructed on the principle reported by Lombardo and Tamburino (1966).

For the determination of electrophoretic mobilities of red cells (and other cells), the only method available hitherto has been the microscopic one, in which the movement of a single cell is observed at a time. These micro-techniques have been used variously in the investigation of electrophoretic behaviour of erythrocytes in electric fields and also changes in the erythrocyte surface charges in certain diseased conditions (Ponder and Ponder, 1955; Hopps and Engles, 1957; Angers and Rottino, 1961; Hayry and Saxén, 1965; Dommert and Dimopoulos, 1965). Although continuous observations have to be made over a considerable period, with the common microscopic apparatus the investigations/

investigations can only be limited to a few single erythrocytes at a time.

Unlike this type of apparatus, the apparatus described below makes it possible to study a whole zone of erythrocytes electrophoretically. Thus, mean values of the migration velocity of the whole erythrocyte suspension under examination could be obtained without having to keep individual cells under continuous observation.

With nearly all the measurable values constant throughout, the only variable is the migration distance. Consequently, this apparatus gave excellent reproducible and quick mobility determinations, which were suitable for the present purpose.

The apparatus, illustrated in Fig.24, consists of a common electrophoresis feeder with a buffer stabiliser, and a migration chamber.

The migration device consists of a wooden support in which 4 hollows, for 4 polythene tubes and vulcathene plugs connected to 2 platinum electrodes (positive and negative), have been made. There is a central wooden support with a groove for the longer U-glass tube. The latter tubes, 3 in all, had a constant internal lumen section of 3 mm. diameter, and were disposed, together with the electrodes, for mobility determinations as shown in Fig.24.

(c) Determination of Electrophoretic Mobility: The first two U-tubes, filled with the electrolytic buffer solution, were used to stabilise the electrical connection between the end polythene tubes and those in the middle. The third end U-tube, also similarly filled, served to shut off the circuit and measure the/

ERYTHROCYTE ELECTROPHORESIS APPARATUS

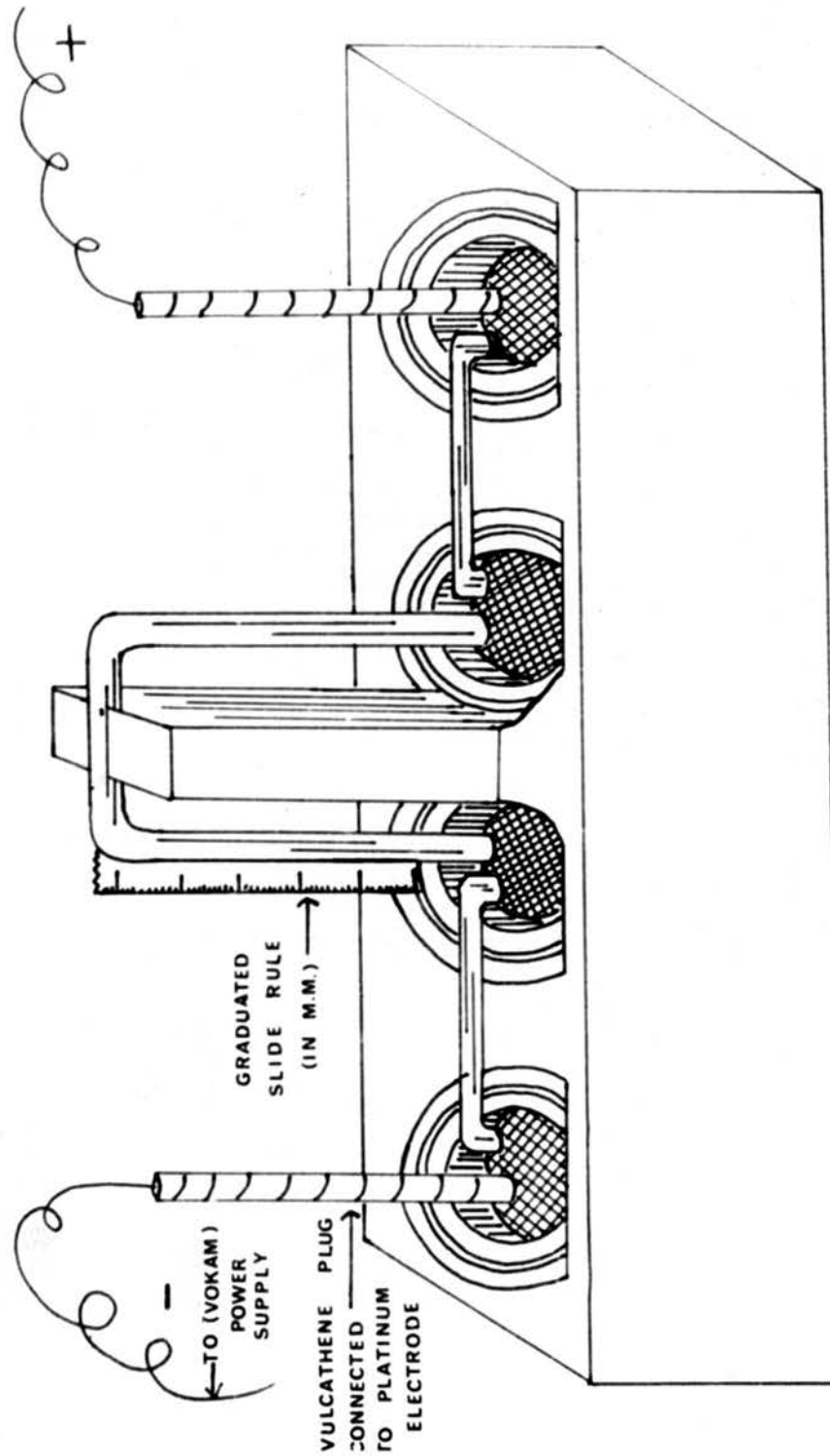


FIGURE 24. Apparatus for Determining the Electrophoretic Mobility of Erythrocytes.

the migration velocity of the erythrocyte column.

Packed erythrocyte sample, after being washed repeatedly with the sucrose-buffer solution; was made up to 50 per cent suspension in the same buffer. Then 2 mls. of the buffer solution were placed in each polythene tube; the U-glass tubes, filled with the same buffer solution, were arranged in the wooden support to stabilise the connection. The two vulcanite plugs with the electrodes were then inserted into the two end polythene tubes when a complete levelling of the buffer solution in the polythene tubes occurred. The central U-glass tube, and the U-tube creating the connection with the negative pole, were carefully taken away. Then 1 ml. of the buffer solution in the middle polythene tube — nearest to the negative pole, and adjacent to the graduated slide rule — was withdrawn and then replaced with an equal volume of the 50 per cent erythrocyte suspension. The connection between all the polythene tubes was then re-established by replacing the first two fluid-filled U-tubes.

Once the electrolyte continuity was established, the erythrocytes, which were suspended in a buffer whose pH was greater than the iso-electric point of the cells, migrated towards the anode by flowing up the vertical stem of the central U-tube. Migration could be followed by observing the column of red cells rise in the graduated U-tube. During this whole electrophoresis period (usually one hour at a time) the voltage and amperage were kept constant. Periodic readings (every 30 minutes) were taken of the distance (in mm.) covered by/

by the cell column.

At the start of the investigations, it was necessary to standardise the apparatus and verify its relative accuracy and reproducibility for determining the change in electrophoretic mobility of erythrocytes.

Using normal erythrocytes, the voltage was fixed at 300 volts and the amperage was 1 milli-amp. The buffer pH was 7.4, with an ionic strength of 0.035.

All experiments were conducted at room temperature, (20°).

(d) Calculation of Electrophoretic Mobility: The mobility was calculated from a formula: $m = \frac{V}{E}$ given by Lombardo and Tamburino (1966), where m is the electrophoretic mobility in microns per second per volt per centimeter, V is the velocity in centimeter per second, and E is the electric field, which is the voltage divided by the length of the electrolytic buffer system in centimeters; E is kept constant throughout the readings.

The migration (electrophoretic) velocity was determined for each particular erythrocyte sample, and since this value is directly proportional to the mobility, the latter value was subsequently readily calculated. Between 6 and 8 migration rates were determined for each type of erythrocyte sample. The degree of variation was calculated as the standard error of the mean mobility for each recorded result.

A simplified form of the formula used in these studies was:

$$m = \left(\frac{ML}{TV} \times 10^4 \right) \mu/\text{sec.}/\text{volt}/\text{cm. where}$$

m = Electrophoretic mobility ($\mu/\text{sec.}/\text{volt}/\text{cm.}$);

/..

m = Electrophoretic mobility (μ /sec./volt/cm.);

M = Migration distance (cm.);

L = Length of electrolytic buffer system (cm.);

T = Time of migration (seconds);

V = Voltage applied (volts)

Experimental Design for Mobility Studies:

- (a) Three breeds of chicken were used to establish a normal erythrocyte migration velocity and electrophoretic mobility for comparison with the sensitized cells. Mobility of erythrocytes of control animals for the infected group of chickens was also similarly determined.
- (b) Mobility determinations were also carried out on in-vitro sensitized chicken erythrocytes obtained from the same animals, which were later infected. Maximal, minimal and 'sham' sensitization of erythrocytes were carried out as described previously (Chapter IV, Part I), and 50 per cent suspension of each batch of cells in 10 per cent sucrose-phosphate buffer was electrophoresed.
- (c) Groups of chicken were infected with S.gallinarum as previously described (Chapter II), and whenever in-vivo erythrocyte sensitization was detected the animal was bled, 50 per cent suspension of red cells prepared and mobility determined.
- (d) Statistical evaluation of mobility values obtained was by means of the students' t-test (Snedecor and Cochran, 1967).

RESULTS

- (1) Demonstration/

(1) Demonstration of in-vivo Sensitizing factor(s) by Immunofluorescence:

The results, illustrated by fluorescent photomicrographs (Figs.25-31), provide direct visual evidence for the presence of both bacterial antigen and an antigen-antibody complex on erythrocyte surface.

Serological specificity of observed immunofluorescence was demonstrated by the complete absence of any fluorescence with the control experiments, an example of which is shown in Fig.25. Thus:

- (a) non-sensitized erythrocytes were not stained by the conjugated antiserum (specific anti-globulin serum);
- (b) staining of in-vivo Type III sensitized cells was inhibited by pre-treatment with unconjugated specific antiglobulin serum;
- (c) conjugated antiglobulin serum, pre-absorbed with specific chicken γ -globulin serum, did not stain preparations of in-vivo Types II and III sensitized cells.
- (d) there was no fluorescence of Type II in-vivo sensitized erythrocytes when these cells were incubated directly with conjugated antiglobulin serum without pre-treatment with specific anti-S.gallinarum polysaccharide serum.

Individual positive cells (Fig.26) showed specific brilliant green fluorescence as a bright rim at the periphery of cells. The same degree of fluorescence was observed with agglutinated erythrocytes (Fig.27). There were no observable distinct differences in the degree of fluorescence obtained with the/

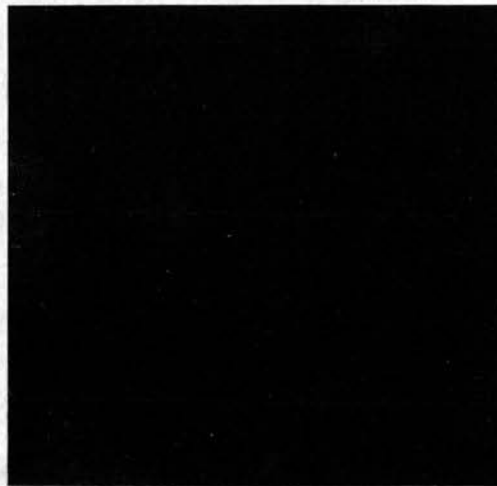


FIGURE 25. Fluorescent micrograph of sham sensitized erythrocytes pre-incubated in anti-S.gallinarum LPS. serum and stained with anti-chicken globulin fluorescein conjugate. Non-fluorescent erythrocytes present, but not visualized in the photograph.
X1800 approx.



FIGURE 26. Fluorescent photo-micrograph of a single in-vitro maximally sensitized erythrocyte, stained with unconjugated specific anti-LPS serum and conjugated anti-chicken globulin serum. Note the bright specific fluorescence of the cell, most marked at the periphery of the cell.

X1800 approx.

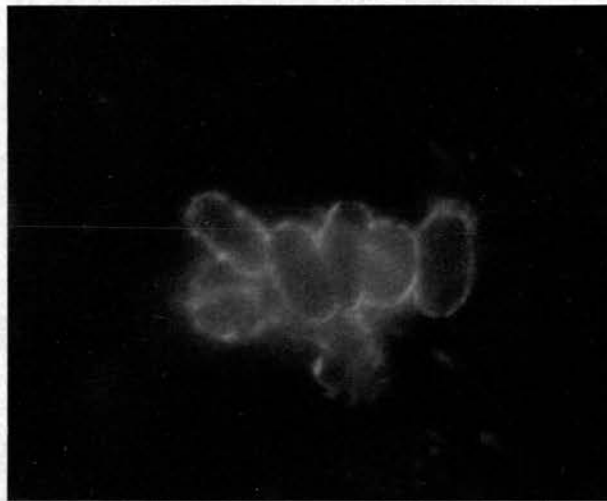


FIGURE 27. Glycerin suspension of an agglutinate of
in-vitro maximally sensitized erythrocytes,
stained as in Fig. 26. Note haemagglutinated
cells showing bright specific fluorescence
similar to Fig. 26.
(Fluorescent micrograph, X1800 approx.)



FIGURE 28. In-vivo Type III sensitized erythrocytes,
demonstrated by staining with fluorescent
anti-chicken globulin serum conjugate.
Note the bright fluorescence of specifically
stained walls of several sensitized erythrocytes.
X1800 approx.

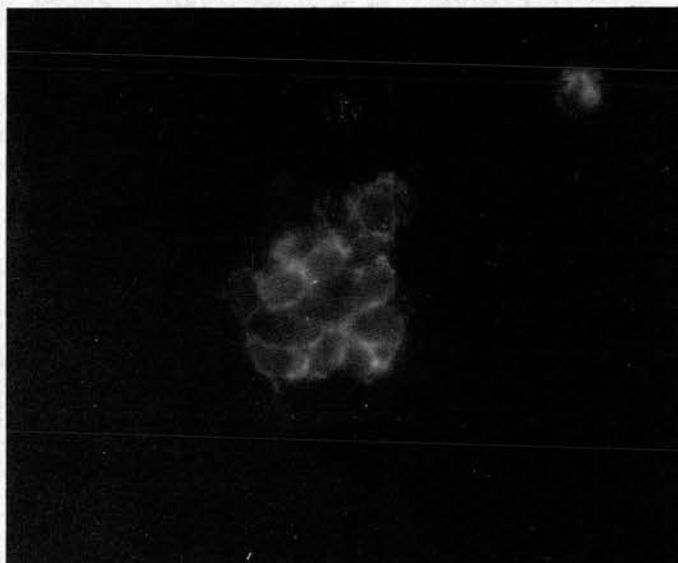


FIGURE 29. Glycerin suspension of an agglutinate of in-vitro maximally sensitized erythrocytes, stained with unconjugated specific anti-LPS serum and conjugated anti-chicken globulin serum. Note large size of agglutinate with slight evidence of the 'trapping' phenomenon. (Fluorescent micrograph, X1500 approx.)



FIGURE 30. Fluorescent micrograph of in-vitro minimally sensitized erythrocytes, stained as in previous Fig. (29). Note single, fairly fluorescent, erythrocytes and absence of specifically stained agglutinates. X1800 approx.

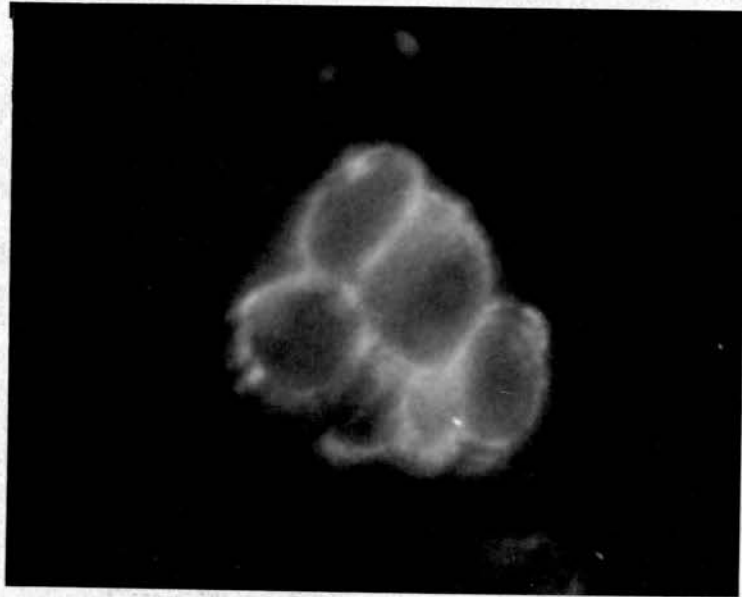


FIGURE 31. In-vivo Type II sensitized erythrocytes,
demonstrated by staining with unconjugated
specific anti-LPS serum and conjugated anti-
chicken globulin serum. Note small size of
fluorescent agglutinate, with well-defined
outlines. Many non-fluorescent individual
erythrocytes present, but few fluorescent
cells seen are not shown in photomicrograph.
X5500 approx.

the two multiple-layer methods. In contrast to the observations of Janković (1959) and Cohen et al., (1960), the addition of a second species (rabbit) specific antiglobulin serum did not significantly enhance the intensity of the reaction. The same degree of staining was achieved with either the two- or three-layer method. Specificity of the three-layer method was sometimes affected by the trapping phenomenon. This phenomenon probably occurred because the second species specific anti-globulin serum was added after agglutinates had already formed, and fluorescent material was thus non-specifically trapped in the interstices of the clumps. With the two-layer and single direct methods, the trapping phenomenon was not a source of confusion. This was demonstrated by the clear, sharp fluorescent outlines of in-vivo Type III erythrocytes (Fig.28). In contrast, samples prepared from in-vitro maximally sensitized cells occasionally produced this phenomenon and therefore necessitated the breaking up of the large agglutinates before suitable photomicrographs could be obtained (Fig.29).

With this technique, small quantities of polysaccharide adsorbed on erythrocyte surface (minimal sensitization) were readily demonstrated (Fig.30). The uniform, homogeneous fluorescence seen was in direct contrast to the equally uniform but heterogeneous fluorescent agglutinates observed with wet preparations from in-vivo Type II sensitized erythrocytes (Fig.31). The latter cells, under the microscope, showed a combination of few individual cells with sharp, clear fluorescent outline, and numerous, but relatively small fluorescent agglutinates with well-defined/

well-defined outlines. On the other hand, in-vitro minimally sensitized cells were seen as a collection of numerous but separate individual fluorescent erythrocytes with only a few or no fluorescent agglutinates (Fig.30).

The fluorescent photomicrographs shown in this study do not indicate the relatively strong fluorescence observed when the wet preparations were being examined under the microscope. Generally, photographic reproduction was difficult since fluorescence in this system was found to fade very quickly.

(2) Extraction of Specific Immune-Complex from Sensitized Erythrocytes:

The results from the in-vitro tests, tabulated in Table 17, demonstrate the success in elution and dissociation of specific antigen (polysaccharide) and homologous antibody from Type III sensitized cells. The results show quite clearly that the direct Coombs positivity, occasionally observed during acute fowl typhoid, is due to the reaction between antibodies specifically directed against an adsorbed bacterial antigen — shown here to be a polysaccharide — and not due to a reaction between the erythrocyte and anti-erythrocyte antibody or non-specifically adsorbed serum globulins.

This important demonstration was supported by the findings that no positive reactions occurred when normal erythrocytes were:

- (a) incubated in the prepared eluate and later titrated against dilutions of specific anti-chicken γ - globulin serum;
- (b) titrated against serial dilutions of erythrocyte - absorbed supernatant/

TABLE 17

DEMONSTRATION OF THE SPECIFICITY AND SEROLOGIC PROPERTIES OF ELUATES FROM IN-VIVO TYPE III SENSITIZED ERYTHROCYTES

TYPE III IN-VIVO SENSITIZED ERYTHROCYTE SAMPLES FROM INFECTED:-	ANTIGLOBULIN HAEMAGGLUTINATION TITRE (EXPRESSED AS THE RECIPROCAL OF FINAL DILUTION OF:)		
	SALM. GALLINARUM ANTISERUM	ELUATE (PRE-ADSORBED WITH PACKED NORMAL RBCs)	ELUATE (PRE-ADSORBED WITH PACKED NORMAL RBCs)
	PRE-ELUTION (DCT)	ADSORPTION OF ELUATE FACTOR WITH NORMAL ERYTHROCYTES (CELLS SUBSEQUENTLY TESTED FOR SENSITIZATION BY ICT)	FACTOR IN ADSORBED ELUATE TITRATED AGAINST POLYSACCHARIDE-SENSITIZED ERYTHROCYTES (ICT)
CHICKEN NO. 109	512	128	16
CHICKEN NO. 28	128	32	8
CHICKEN NO. 67	256	32	16
NORMAL CHICKEN ERYTHROCYTES	0	0	0
OTHER CONTROL EXPERIMENTS CONDUCTED: FIRSTLY, ALL SHOWED THAT NEITHER THE SPECIFIC SALM. GALLINARUM ANTISERUM NOR THE SPECIFIC RABBIT ANTI-CHICKEN ERYTHROCYTE SERUM ALONE WAS CAPABLE OF CAUSING NORMAL CHICKEN ERYTHROCYTES TO AGGLUTINATE. SECONDLY, NONE OF THE SERA FROM CHICKENS FROM WHICH TYPE III IN-VIVO SENSITIZED ERYTHROCYTES HAD BEEN COLLECTED WERE SHOWN TO HAVE ANY SPECIFIC ACTIVITY (AUTO ANTIBODY) AGAINST NORMAL CHICKEN ERYTHROCYTES FROM DIFFERENT BREEDS BY THE DCT METHOD.			

supernatant from the eluate;

(c) either incubated with the specific S.gallinarum antiserum or directly with the specific rabbit anti-chicken γ -globulin serum;

(d) tested against sera of chickens — from which in-vivo Type III sensitization had been detected — for antibody activity (auto-antibodies).

The specificity and identity of the eluate constituents were demonstrated by the ability of one of the constituents to adsorb on to a normal erythrocyte, and subsequently to agglutinate in the presence of specific anti-polysaccharide and anti-chicken γ -globulin sera. The other factor in the absorbed supernatant was also found to specifically agglutinate in-vitro maximally sensitized erythrocytes in the presence of specific anti-chicken γ -globulin serum. This clearly indicated the presence of specific antiglobulin haemagglutinins in the eluate.

It was also observed that only relatively small amounts of specific eluate factors could be extracted, as shown by the overall low antiglobulin haemagglutinin titres (Table 17). Surprisingly, greater amounts of homologous antibody could not be demonstrated in the red cell eluates from chickens with comparatively high titres of antiglobulin haemagglutination as a result of in-vivo Type III sensitization (See chicken numbers 109 and 67 of Table 17). Much smaller quantities of components, specifically derived from S.gallinarum, were also shown to be present in the eluates from the sensitized erythrocytes — a titre/

titre of 1:128 being the highest recorded.

The specificity of the eluate factors was also demonstrated by the absence of any cross-reactivity with both an E.coli polysaccharide-sensitized erythrocytes and homologous polysaccharide antiserum.

(3) Effect of Sensitization on Electrophoretic Mobility of Erythrocytes:

The overall results of mobility measurements are summarized in Table 18. It was concluded from these results that sensitization of the erythrocytes results in significant reduction in the electrophoretic mobility of the cells.

(a) Electrophoretic Mobilities After in-vitro Treatment of Erythrocytes: Table 18 shows that there was no significant difference between the mobilities of the sham sensitized and normal erythrocytes ($p > 0.2$), indicating that neither the process of incubation, temperature treatment, nor repeated washings has any significant effect on the negative electrostatic charges on the cell surface. However, in-vitro sensitization of erythrocytes with polysaccharide caused significant reduction in the electrophoretic mobility of the cells, the degree of reduction depending on the amount of polysaccharide adsorbed on erythrocyte surface. Thus, cells minimally sensitized with polysaccharide had a significantly lower ($p < 0.05$) electrophoretic mobility than cells subjected to the same in-vitro procedure for sensitization but without the polysaccharide (that is, sham sensitization). On the other hand, compared with either sham sensitized or normal erythrocytes/

TABLE 18

SUMMARY OF ERYTHROCYTE ELECTROPHORETIC MOBILITY VALUES FROM NORMAL AND INFECTED CHICKENS

STATUS OF CHICKEN ERYTHROCYTES ELECTROPHORESSED	TOTAL NUMBER OF OBSERVATIONS (DETERMINATIONS) (n)	MEAN ELECTROPHORETIC VELOCITY (IN mm/30 MINUTES)	ELECTROPHORETIC MOBILITY ⁺ (IN μ /SEC/VOLT/CM)
NORMAL AND 'NON-SENSITIZED' (FROM THREE DIFFERENT BREEDS)	20	17.0	1.05 \pm 0.006
'SHAM' SENSITIZED	7	16.5	1.03 \pm 0.011*
IN-VITRO MINIMALLY SENSITIZED	7	15.0	0.94 \pm 0.027#
IN-VITRO MAXIMALLY SENSITIZED	7	8.0	0.50 \pm 0.026###
IN-VIVO SENSITIZED (TYPE II)	8	10.5	0.66 \pm 0.064***
IN-VIVO SENSITIZED (TYPE III)	8	9.5	0.59 \pm 0.044***
+ RESULTS EXPRESSED AS MEAN \pm STANDARD ERROR OF MEAN			
* = DENOTES NO STATISTICALLY SIGNIFICANT DIFFERENCE, AT P > 0.2, FROM THE NORMAL VALUE # = DENOTES STATISTICALLY SIGNIFICANT DIFFERENCE, AT P < 0.05, FROM 'SHAM' SENS. VALUE ### = DENOTES VERY HIGHLY STATISTICALLY SIGNIFICANT DIFFERENCE, AT P < 0.001, FROM 'SHAM' SENS. VALUE *** = DENOTES VERY HIGHLY STATISTICALLY SIGNIFICANT DIFFERENCE, AT P < 0.001, FROM NORMAL VALUE			

erythrocytes, the electrophoretic mobility of maximally sensitized cells was very markedly decreased ($p < 0.001$).

It has also been found that increasing the pH of the suspending buffer (for example, up to 8.2) did not influence appreciably the final electrophoretic mobilities. This indicated that once the pH of the buffer has exceeded the iso-electric point of the migrating cells, these cells did not migrate any faster.

(b) Electrophoretic Mobility of *in-vivo* Sensitized Erythrocytes:

A very highly significant retardation of the mobility ($p < 0.001$) was observed with each type of *in-vivo* sensitized cells, as compared with the normal values (Table 18). The Table also shows that, regardless of the type of *in-vivo* sensitization, the decrease in mobility was very substantial ($p < 0.001$), even though they were not as greatly reduced as when the cells were maximally sensitized *in-vitro*. However, there were also highly significant differences ($p < 0.01$) between cells sensitized *in-vivo* with only the bacterial antigen (polysaccharide)(Type II) and those with antigen-antibody complex (Type III),

DISCUSSION

The above studies have demonstrated quite conclusively that the *in-vivo* erythrocyte sensitizing factors are bacterial polysaccharide and specific homologous antibody.

It is well known that lipo-polysaccharides (LPS) from gram-negative bacteria have the property of inducing characteristic febrile (Thomas, 1954; Atkins, 1960; Keene et al., 1961; Watson and Kim, 1963; Kimball and Wolff, 1967) and leucocytic/

leucocytic (Wood, 1958; Atkins, 1960; Stetson, 1961; Yoffey et al., 1963; Visscher, 1965) responses in animals, especially in rabbits, into which they have been injected. While these are not properties confined to LPS, the fact that such small quantities of LPS evoke these characteristic responses places them in an unique class (Bennet and Cluff, 1957; Zweifach and Janoff, 1965; Nowotny, 1969). In the present experiments, rabbits inoculated with in-vivo Type II sensitized erythrocytes responded with typical biphasic pyrogenic responses (Fig.23). A similar febrile response pattern was also observed when erythrocytes, sensitized in-vitro — either minimally or maximally — with LPS, were injected into rabbits (Fig.22). In contrast, in-vivo Type III sensitized erythrocytes and cells minimally sensitized in-vitro with LPS and then incubated with homologous antiserum, elicited only monophasic pyrogenic responses (Figs.23B and 22C respectively). The latter results indicate that the adsorption of globulin by erythrocytes, previously sensitized with polysaccharide, inhibits one of the fever peaks of the otherwise biphasic response pattern. However, the finding that rabbits injected with in-vivo Type II sensitized erythrocytes respond with typical biphasic fever pattern could reasonably be attributed to an adsorbed bacterial polysaccharide. Such an interpretation, while likely, is not conclusive in view of the demonstration by Grey, Briggs and Farr (1961) that classical antigen-antibody reactions will produce biphasic fever responses in rabbits, and the finding of Jandl (1957) and Jandl and Tomlinson (1958) that immune haemolysis in-vivo is accompanied by marked pyrogenic/

pyrogenic and profound leucopaenic responses analogous to reactions to bacterial endotoxins. However, the latter authors found that haemolysis induced by non-immunologic methods did not result in fever or leucopaenia. In any event, these present studies have shown that rabbit responses to injection of in-vivo Type II sensitized and normal, non-sensitized chicken erythrocytes are clearly biphasic and monophasic respectively. Moreover, it has recently been shown that the pyrogenic response to the classical antigen-antibody reaction is not biphasic, but monophasic (Nott and Wolff, 1966). These authors also reported that in contrast to the near complete absence of a latent period normally seen in endotoxin fever curves, the latent period before temperature rise following antigen-antibody injection is usually very long. The conclusion that the sensitizing factor of the in-vivo Type II erythrocyte may be a polysaccharide is supported by the finding by Sell and Braude, (1961) that rabbits intravenously injected with E.coli endotoxin-sensitized erythrocytes developed typical biphasic fever curves, whereas control rabbits given non-sensitized erythrocytes only responded with low-grade monophasic temperature elevations.

Unlike the pyrogenic response, the leucocytic response of the rabbit to intravenously injected sensitized erythrocytes did not follow the classical pattern of initial heterophile leucopaenia (neutropaenia) caused by endotoxin, followed at variable intervals by leucocytosis involving largely immature granulocytic forms — neutrophilia (Atkins, 1960; Visscher, 1965; Chervenick, Boggs, Marsh, Cartwright and Wintrobe/

Wintrobe, 1967). With the exception of erythrocytes sensitized maximally in-vitro with specific endotoxin, none of the sensitized erythrocyte preparations caused the initial leucopaenia, but 24 hours later all injections were followed by various degrees of leucocytosis, with only marginal or no response after injection of in-vivo Type III sensitized erythrocytes (Table 11). The failure to detect initial neutropaenia may be explained by the finding that the characteristic pattern of leucocytic response to endotoxin administration is dose-dependent (Atkins, 1960; Boggs, Cartwright and Wintrobe, 1966; Chervenick et al., 1967). Furthermore, the finding that neutropaenia is not observed after small doses of endotoxin in the rabbit (Farr and Le Quire, 1950; Olitzki, 1959) or the monkey (Sheagren, Wolff and Shulman, 1967) but that these injections were followed later by different degrees of neutrophilia, lend further convincing support to the suggestion that the absence of initial leucopaenia in these studies was due to the relative smallness of the total dose of Type II in-vivo sensitized erythrocytes — and hence polysaccharide — injected. In addition, even though previous studies by Smith, Alderman and Cornfield, (1961), had shown no clear relationship between neutrophil changes and dose of endotoxin administered in the mouse, recent studies by Chervenick et al., (1967) have demonstrated that small doses of endotoxin in the mice induce only neutrophilia, without preliminary neutropaenia.

Further evidence for S.gallinarum polysaccharide as the erythrocyte sensitizing factor was afforded by the specific haemagglutination studies. These showed that cells obtained from infected/

infected animals were capable of inducing high titres of specific anti-S.gallinarum polysaccharide antibodies in the rabbit. This was of interest, in view of the fact that the present studies have indicated the presence of very small amounts of polysaccharide on the erythrocyte surface as a result of infection (Tables 14 and 15). However, Bokkenheuser and Koornhof (1959) have demonstrated that bacterial polysaccharide adsorbed on erythrocytes is highly immunogenic and Landy, Johnson, Webster and Sagin (1955) have shown that the antigenicity of endotoxin in mice, rabbits and man, when compared with that of intact gram-negative bacteria, is considerably greater than the organism from which it was derived. For example, in rabbits, a single intravenous injection of a small amount of purified S.typhosa endotoxin (0.001 µgm. per adult rabbit) was found to evoke a high level of haemagglutinins, and the haemagglutinin levels so evoked were shown to be in proportion to the endotoxin dose.

Whilst the pyrogenic and leucocytic effects of injected in-vivo sensitized (Type II) erythrocytes only indicated the probable presence of a pyrogen or a lipo-polysaccharide adsorbed on erythrocytes, the finding that these cells induced specific anti-S.gallinarum polysaccharide antibodies after injection (Tables 12-13) makes it more likely that the sensitizing factor is a specific polysaccharide which has been adsorbed in-vivo by the erythrocytes during infection. Furthermore, using immunoelectrophoretically tested specific antisera, the specificities of the adsorbed bacterial antigen and the antigen-antibody complex, of respectively the in-vivo Types II and III sensitized cells/

cells, were confirmed by positive indirect and direct Coombs' tests (Table 16).

Immunocytochemical evidence for specific polysaccharide and specific homologous antibody as the in-vivo sensitizing factors was also provided by the results of the immunofluorescent experiments. A variety of specific antigen-antibody reactions involving tissues, microorganisms and viruses have been readily demonstrated by means of the fluorescent antibody technique introduced by Coons and Kaplan (1950). The visualization of antigen-antibody systems, involving antigenic components of erythrocytes by this method, has always been either unsuccessful or difficult (Alexander, 1958; Whitaker et al., 1959; Cohen et al., 1960; Lee and Vazquez, 1962). Properly controlled, however, the immunofluorescent method offers an ideal procedure for the study of specificity of erythrocyte antigen-antibody systems. In the results reported here, fluorescence of agglutinates or individual sensitized erythrocytes was observed only when the erythrocyte preparations were exposed to fluorescent conjugates containing species specific immunoglobulins. The problem of specificity in these results was adequately controlled by the inclusion in all experiments of negative control experiments involving non-sensitized and sensitized erythrocytes, and blocking experiments with conjugated and unconjugated specific antisera. Serological specificity of observed immunofluorescence was subsequently demonstrated by the complete absence of any fluorescence with the control experiments. Thus, the presence or absence of fluorescence, at least under the conditions of the/

the present experiments, was clearly an all-or-none phenomenon and therefore the problem of having to discriminate between weak, strong and non-specific fluorescence as a means of defining specific reactions, did not arise. Similar procedures were used by Janković (1959), Cohen et al., (1960), Lee and Vazquez (1962) and Johnson et al., (1965) in studies of red cell antigen-antibody systems.

The present results have demonstrated that even very small quantities of lipo-polysaccharide adsorbed on erythrocyte surface can be easily shown visually. The most interesting aspect of the use of this method has been — apart from confirming the specificity of the adsorbed bacterial antigen — the actual visual demonstration of the sensitizing factors in situ. Successful application of this technique has provided, both an accurate revelation of the sites of specific immune reactions and direct immunocytochemical evidence for the presence of specific lipo-polysaccharide and homologous antibody at the surface of sensitized erythrocytes from chickens with acute fowl typhoid.

In spite of the biologic, serologic and immunocytochemical demonstrations of the specificity of the antibody in Type III sensitization, the possibility also existed that some of these in-vivo Type III sensitized erythrocytes may, in fact, have resulted from non-specific adsorption of serum globulins (Stratton and Jones, 1955; Jandl, 1960; Warner, 1962; Sutherland et al., 1963; Jensen, 1965), or the specific reaction between immunoglobulins/

globulins and erythrocyte surface antigens (Dacie, 1950; 1963; 1968; Dacie et al., 1957). The likelihood of this happening is suggested by the finding of Warner (1962) that erythrocytes of chickens between the ages of one week and 4 months have a coating of globulins detectable by the anti-globulin reaction. The factor responsible for this coating was shown to be normally present in the serum. However, this finding may be explained by observations made in the course of the present studies which clearly showed that such non-specific Coombs positive reactions could be obtained with incompletely absorbed "crude" rabbit anti-chicken whole serum (Chapter III) and also with normal chicken serum containing high levels of iso-haemagglutinins. Therefore, in an attempt to definitely characterize the in-vivo Type III sensitizing factor(s), various experiments were conducted in which the sensitizing factor(s) were eluted, dissociated and finally, serologically tested and identified. The results of these extraction experiments, summarized in Table 17, clearly show that the in-vivo Type III sensitizing factors consist of a mixture of a specific polysaccharide and homologous antibody. Therefore, the direct Coombs positivity observed during acute fowl typhoid is the result of the reaction between antibodies directed specifically against an adsorbed S.gallinarum polysaccharide, and is not due to a reaction between erythrocyte and anti-erythrocyte antibodies or non-specific globulin adsorption. In the present studies, the fact that relatively small amounts of specific eluate factors were obtained was probably more a reflection/

reflection of the sensitivity of the tests and the experimental conditions of the techniques used for the elution, dissociation and serologic identification of the eluate factors, rather than due to any lack of specificity in the eluted factors. Indeed, difficulties encountered by Springer and Horton (1964) in demonstrating elution of significant amounts of the antigen and antibody from erythrocytes sensitized in-vitro with E. coli polysaccharide and homologous antibody were attributed to the general insensitivity of the Landsteiner-Miller elution method (Landsteiner and Miller, 1925), which was used in the present studies. On the other hand, Young and associates (Young et al., 1962), using the same method but combined with Weiner's alcohol precipitation technique (Weiner, 1957), were easily able to elute from ostensibly Type III in-vivo sensitized erythrocytes significant amounts of bacterial substances — cross-reactive with the infecting E.coli strains — which had been acquired in-vivo by the erythrocytes of infants with enteritis. The small amounts of the two components extracted may also be explained by the fact that, in conformity with the concept of heterogeneity of antibodies with respect of their avidity for the antigen (Pauling et al., 1944; Karush, 1959; Talmage, 1959) hapten or antigen molecules (of polysaccharide) may be removed mainly from antibody combining sites with low affinities, and therefore weakly or loosely bound. The corollary of this effect is that equally, some of the strongly binding, high-affinity antibody in the eluate will remain attached to the eluted antigen/

antigen, thereby effectively blocking some of its antibody combining sites.

As a logical follow up to the demonstration of in-vivo erythrocyte sensitization, possible changes in the electrostatic charge on the surface of these sensitized erythrocytes were determined. The results of these changes, reflected in highly significant decreases in the electrophoretic mobility rates of the sensitized erythrocytes, indicate that during acute fowl typhoid the erythrocytes are altered electrostatically. The data clearly indicates that these significant decreases in the electrophoretic mobility were the direct result of the changes induced by the adsorption of polysaccharide or the specific bacterial antigen and homologous antibody. In support of this conclusion is the finding that the erythrocytes of sludged blood (in-vivo agglutinated erythrocytes) have visible surface changes (Knisely and Block, 1942; Knisely, et al., 1947; Knisely, 1959), and that such cells have significantly decreased electrophoretic mobilities (Redmond, 1948; Bellis and Snow, 1950).

The finding of marked retardation in the electrophoretic mobility of in-vivo sensitized erythrocytes of chickens with acute fowl typhoid, considered on its own is not unusual, in view of the finding by various authors that certain haematologic disorders in birds decrease the mobility of the erythrocytes (Brown, 1933; Findlay and Brown, 1934; Redmond, 1948). However, all these cases involved parasitic protozoan blood infections, and the mobility changes might have been due to the/

the direct consequence of changes induced in the erythrocyte surface negative charges by parasitization of the cells, or adsorption of proteins on the cell surfaces. Nonetheless, the fact still remains that alteration of erythrocyte surface by various infectious organisms greatly affect its mobility (Ponder and Ponder, 1955; Hopps and Engles, 1957; Rottino and Angers, 1962; Häyry and Saxén, 1965, Dommert and Dimopoulos, 1965; 1966; 1967).

In the present studies, highly significant ($p < 0.01$) differences between the mobilities of erythrocytes sensitized in-vivo with only the polysaccharide (Type II) and those with the antigen-antibody complex (Type III) were observed. In addition, the mobility of the latter sensitized erythrocytes was also very much decreased ($p < 0.001$) as compared with that of normal or sham sensitized erythrocytes. These observations could be explained by the finding that in-vivo adsorption of proteins by erythrocytes during certain infections greatly reduces the electrophoretic mobility (Brown, 1933; Redmond, 1948; Hartman and Nungester, 1953; Creger, Tulley and Hansen, 1956; Shideler, 1960; Rottino and Angers, 1962). Furthermore, the recent demonstration by Sundaram, Phondke and Ambrose (1967) and Diengdoh and Turk (1968) that the occurrence of a specific antigen-antibody reaction on cell surface results in very decreased electrophoretic mobility of such cells attests to the validity of the above explanation; it also explains the observations made when in-vivo Type III erythrocytes were electrophoresed. The variations in the decrease of mobility rates/

rates were found to parallel the types of in-vivo erythrocyte sensitization, thus probably indicating that the in-vivo alteration of the erythrocyte surface is a stepwise change — the immune reaction sequence being initiated by the adsorption of polysaccharide by the erythrocytes, followed later by reaction with the homologous antibody.

The finding of general retardation of electrophoretic mobility of in-vivo sensitized erythrocytes suggests that the surface charge of erythrocytes is greatly influenced by the immune reactions occurring on surface of cells during acute fowl typhoid, and the method for estimating electrophoretic mobility can be used successfully to demonstrate such cellular immune changes during other acute bacterial infections.

CHAPTER VTHE ANAEMIA OF FOWL TYPHOID: ERYTHROCYTE SURVIVAL
STUDIES USING RADIOCHROMIUMINTRODUCTION

The occurrence of a clinical anaemia during the course of fowl typhoid has long been recognised (Ward and Gallagher, 1920; Cook and Dearstyne, 1934; Gordon and Brander, 1942; Wilson, 1946; Menzies, 1947; Hungerford, 1951; Smith, 1955; Buxton, 1957; 1959b; Hagan and Bruner, 1961; Hall, 1965; Smits, 1966). Previous results have defined the character and extent of this anaemia under controlled experimental conditions (Chapters II and III). However, standard haematological techniques do not provide any indication of the kinetics of erythrocyte turnover and survival under the conditions of infection. Such observations would provide direct evidence that the lesion was caused by an increased destruction of erythrocytes.

The most accurate technique for the estimation of red cell life-span and the study of erythrocyte survival in haematologic diseases, is the radiochromium method (Ebaugh, Emerson and Ross, 1953; Necheles, Weinstein and Le Roy, 1953; Mollison and Veall, 1955; Donahue, Motulsky, Giblett, Pirzio-Biroli, Viranuvatti and Finch, 1955; Hughes-Jones and Mollison, 1956; Mollison, 1961; 1967). Since the use of ^{51}Cr for the determination of the life-span of chicken erythrocytes has been demonstrated by Rodnan, Ebaugh and Fox (1957), Jaffe and Hopkins (1961), Walter, Brake and Selby (1965) and Wels, Schrappauf and Horn (1967), it was decided to apply this technique to the study of the anaemia associated with fowl/

fowl typhoid.

The present studies were designed with the following objectives:

- (1) To obtain direct evidence of haemolysis (increased destruction of erythrocytes).
- (2) To demonstrate whether such haemolysis could account for the severity of the anaemia observed.
- (3) To estimate the erythrocyte survival time which should permit an accurate expression of the rate of erythrocyte destruction relative to the normal value, and to provide evidence of any shortened erythrocyte life-span as a result of infection.
- (4) To investigate the nature and type of the in-vivo haemolytic mechanism by studying the pattern of departure from the normal rate of erythrocyte elimination consequent upon infection.
- (5) To observe and evaluate the effects of various treatments of the host on the rates of survival and destruction of labelled erythrocytes.
- (6) To study the effect of various treatments of erythrocytes in normal and immune hosts.
- (7) To ascertain the origin of the destructive factors responsible for the initiation of the haemolytic crisis; that is, delineation of the intra- and extra-corpuscular defects (Veall and Vetter, 1958; Dacie and Lewis, 1968).
- (8) To establish the sites of removal and destruction of erythrocytes

MATERIALS AND METHODS

Experimental Animals: Two breeds of chickens were used. In the first/

first group approximately 60 white-link chickens, aged between 10 and 12 weeks and of mixed sex, were used for the injection studies. The second group of chickens were used for all the S.gallinarum infection studies, and this consisted of 6-month old 'Hybrid 66' hens (Chunky-Chick Ltd.).

Preparation of S.gallinarum Hyperimmune Serum: Specific hyperimmune sera were prepared in chickens against S.gallinarum strain being used in these studies by a multiple inoculation schedule. A washed, heat-killed (100°C for 60 minutes) suspension, containing approximately 1.5×10^9 organisms per ml. was injected intravenously. The animals were bled 10 days afterwards, and then re-immunized by injecting the same dose into each pectoral muscle. They were bled again 7 days after these intramuscular injections. The sera from these 2 bleedings were pooled, the O-antibody level assayed by haemagglutination, and stored. (Samples of this serum were fractionated on Sephadex G200 and pooled fractions were later examined by immunoelectrophoresis (Chapter III), and found to contain essentially IgM antibodies, with small but significant levels of incomplete IgG antibodies).

S.gallinarum Lipo-polysaccharide (LPS) Preparation: A purified, LPS was prepared by the technique outlined in Chapter III.

Haematological Procedures: Methods for packed cell volume (PCV) (determined on all serial blood samples withdrawn for isotope counting) and haemoglobin concentrations (estimated in all animals at the beginning and end of each experiment) have been previously described (Chapter II).

Erythrocyte Survival Studies:

(a)/

(a) Radiochromium Labelling: The in-vitro labelling technique, with ^{51}Cr as a sterile isotonic sodium chromate ($\text{Na}_2^{51}\text{CrO}_4$) solution (Radio-chemical Centre, Amersham), was used.

The procedure for labelling was a modification of the method described by Jaffe and Hopkins (1961): 6 mls. of blood were withdrawn aseptically by syringe through the wing vein into a quarter of its volume of acid citrate-dextrose solution (ACD), containing 2 gms. of disodium citrate monohydrate and 3 gms. dextrose anhydrous in 120 mls. of distilled water; pH was 5.5. The erythrocytes were packed by gentle centrifugation at 1,000 r.p.m. for 30 minutes, and the plasma-ACD supernatant was removed and made up to 3 per cent plasma with sterile isotonic saline.

A dose of $\text{Na}_2^{51}\text{CrO}_4$, corresponding to 3 to 4 μC per ml. of blood, was added to packed erythrocytes, mixed and incubated for 30 minutes at 42°C , with regular gentle mixing every 5 minutes. (The high specific activity, corresponding to 100 mC per mg. ^{51}Cr , was such that the radiochromium added did not exceed 10 to 20 μgm ^{51}Cr per ml. of red cells (Ebaugh *et al.*, 1953; Donahue *et al.*, 1955; Stohlman and Schneiderman, 1956). The labelled cells were then packed by gentle centrifugation, supernatant removed, and then washed once with 20 volumes of 3 per cent autologous plasma-saline (preliminary pilot experiments showed that this procedure resulted in 85 to 95 per cent incorporation of the isotope in the erythrocytes).

(b) Preparation of Standard and Injection Procedure: A known volume of the reconstituted labelled cells (0.5 ml.) was accurately measured out from a calibrated syringe for the preparation of a standard. This was made up to 20 mls. haemolysate with distilled water./

water. A uniform, homogeneous counting geometry was obtained by the addition of a knife point of saponin to the haemolysate (Van Kampen and Heerspink, 1961). An equal small volume (1 ml.) of this standard preparation was counted each time with the serial blood sample.

For all injections, an accurately measured volume (5 mls.) of the remaining labelled cells, which had previously been resuspended in 3 per cent plasma saline to the original volume of the blood withdrawn, were transfused via the wing vein without delay.

(c) Radioactive Counting of Blood Samples: To obviate cleansing of glassware, disposable Sequestrene (EDTA) tubes (Styne Laboratories) and plain plastic, screw-top vials (Shipton Plastics, Middlesex) were used, respectively, for serial blood collection and counting.

After injection of labelled cells, samples not exceeding 1 ml. were taken rapidly into a sterile syringe which had been wetted with EDTA and quickly delivered into the disposable Sequestrene tube. (Samples were taken after 10, 30 and 60 minutes after transfusion if whole blood and erythrocyte volumes were to be determined). For routine plotting of the ^{51}Cr clearance rates (survival curves), and unless otherwise stated to the contrary, the zero day (100 per cent survival) was taken as the 24 hour sample to establish the baseline (Necheles et al., 1953; Veall and Vetter, 1958; Dacie and Lewis, 1968).

For normal survival studies it was necessary to bleed the animals no more frequently than daily for 3 days and thereafter at intervals of 3 or 4 days. Daily samples were, however, needed for the infection studies.

(d) /

(d) Preparation of haemolysate for counting: For normal survival studies 1 ml. of accurately measured blood sample was pipetted into the disposable counting vial, and haemolysed by the addition of a knife point of saponin. (For all other survival studies, 0.5 ml. of the blood sample was accurately measured, lysed with an equal amount of distilled water and a sprinkling of saponin). The top of the vial was screwed down and the activity subsequently counted.

(e) Counting of Radioactivity: Counting of activity in the prepared haemolysate was carried out on a thallium-activated sodium iodide well-type crystal scintillation counter (Panax Equipment, Ltd.) connected through a photomultiplier to an amplifier and a scaler which displays the number of impulses in a pre-set time. The counter was calibrated before use to ensure high efficiency (Joske et al., 1956; Dacie and Lewis, 1968), and the Extra high tension unit (EHT) and Discriminator Bias (DB) were fixed at 1200 and 7.5 volts respectively. A series of background counting readings — 1000 counts per counting — were taken and the mean determined. Determination of sample radioactivity was, however, based on the mean of 3 minimum counts of 10,000. Correction for physical decay was ensured by making these comparative measurements: the counting of an equal volume of the standard each time and soon before, and after, the counting of the Day t sample (Weinstein and Le Roy, 1953).

After correcting both readings for background activity, the sample radioactivity was expressed as 'counts per second per ml. whole blood'. This method of measurement of radioactivity had been recommended by many investigators as the ideal procedure for disorders in which the erythrocyte volume is changing/

changing rapidly, for example, in haemolytic syndromes where the rate of erythrocyte destruction greatly exceeds the rate of production (Read, Wilson and Gardner, 1954; Marvin and Lucy, 1957; Veall and Vetter, 1958; Stohlman, 1961; Blahd, 1962; Brecher, 1967; Dacie and Lewis, 1968). However, where there were no significant changes in the total erythrocyte numbers, the activity was expressed as counts per second per unit PCV.

The final activity of all these sample counts were finally expressed as the ratio of the activity in each sample to the activity of the standard, and this corrected for physical decay, as mentioned above. The disappearance of radioactivity (labelled cells) from the circulation was then calculated for each sample, taking either the 24-hour or 10 minute value, where appropriate, as the 100 per cent survival (Zero Day).

The findings that the rates of ^{51}Cr elution from normal and abnormal cells in many haematologic disorders are the same (Read et al., 1954) and that correction for elution is unnecessary in disorders where erythrocyte destruction is abnormally high and rapid (Necheles et al., 1953; Donahue et al., 1955; Hughes-Jones and Mollison, 1956; Cline and Berlin, 1963; Dacie and Lewis, 1968) led to the decision not to correct for ^{51}Cr elution in the present studies. In any case, the present studies consisted essentially of comparative measurements.

(f) Calculation of Erythrocyte Survival: Sample activity, expressed as counts per second per unit of whole blood (or per unit PCV), was calculated as a percentage of the activity remaining on Day-0 (Zero Day). For subsequent Day-t measurements, the percentage ^{51}Cr survival was given by the ratio:

Counts/

$$\frac{\text{Counts/sec./ml. blood on Day-t} \times 100}{\text{" " " " " Day-O}}$$

These values were subsequently plotted graphically against time on an arithmetic graph.

Individual elimination curves were interpreted according to the regularity and randomness of the erythrocyte destruction. Erythrocyte survival was expressed as the Half-Life ($T_{\frac{1}{2}}^{51}\text{Cr}$) — the time when 50 per cent of the activity still remained in the circulation. In spite of the absence of any physico-biological significance in the latter parameter (Bergner, 1965), it was, however, included here for comparison with the more statistically significant mean red cell life (MCL) and maximum red cell life to conform to accepted usage. Where appropriate, the MCL — the average life-span of the whole red cell population — was estimated by extrapolating a tangent at the initial linear portion of the curve to the abscissa, as recommended by Dornhorst (1956), Marvin and Lucy (1957), Lewis, Szur and Dacie (1960) and Mollison (1967). MCL estimation depends on the type of the survival curve obtained, whilst the maximum cell life is readily estimated as the point of intercept of the curve with the abscissa (Marvin and Lucy, 1957).

Apparent survival curves (Necheles et al., 1953) were also compared graphically.

(g) Determination of Residual Organ Radioactivity: At the termination of the experiment, the animal was bled through the jugular vein under light anaesthesia to remove the remaining circulation radioactivity (Braude et al., 1955). The animal was then sacrificed and the spleen and liver removed, weighed and soaked in water at 4°C until the washings contained no apparent haemoglobin. This procedure was necessary to ensure that the presence/

presence of radioactivity in an organ was not that of the blood contained within it (Braude et al., 1955). (Animals which had died as a result of infection were, however, immediately autopsied, and the organs removed and likewise processed as described above.)

1 gm-quantities of the organs were counted after being digested with hot, concentrated nitric acid, according to the method described by Burton and Mollison (1968). Each gm-aliquot was dissolved in 10 mls. of nitric acid before 2.5 mls. of this were counted; the total (absolute) activity in each organ (net weight) was then calculated. Corrections for decay were made as before.

Finally, the distribution of the isotope was counted as a fraction or percentage of the organs' specific radioactivity to the total injected radioactivity. The problems of variable quenching (gamma ray absorption) by different tissues, and other interfering factors, have not been surmounted by this procedure. However, by combining the various techniques described above, it is probable that a reasonably accurate organ activity estimations were obtained.

The total, initial radioactivity was calculated by the method described by Veall and Vetter (1958).

Design of Experiments:

Erythrocyte survival studies were performed by separating the overall experiments into 2 sections:

(1) Studies in Non-Infected Chickens

White-Link chickens were used; they were divided into 4 groups:

Group I:- Normal Erythrocyte Survival Studies: The animals were/

were auto-transfused with labelled cells and survivals were determined to establish the normal rate and pattern of elimination for purposes of comparison later on. Total blood and erythrocyte volumes were estimated by the procedure outlined by Veall and Vetter (1958). The activity in these studies were expressed as counts per second per unit haematocrit (PCV).

Group II:- Erythrocyte Survival Studies following Induced Haemolysis: (See Chapter VII). Each one of these animals was auto-transfused with the usual dose of labelled-erythrocytes, and the survival rate determined for at least one week. Afterwards, each animal was intravenously injected with a sensitizing dose of S. gallinarum endotoxin (40 mg per Kgm.) (This dose of endotoxin has been found to sensitize the erythrocyte in-vivo — Chapter VII). Subsequently, the elimination of these in-vivo sensitized, labelled cells was followed by serial radioactivity determinations.

Control experiments for this group consisted of a reversal of the injection procedures for the test experiments:

(a) autologous labelled, but non-sensitized, cells were transfused either 6 or 24 hours after the injection of 40 mg. per Kgm. endotoxin.

(b) Chickens were given sterile saline in place of endotoxin, but injections were in the same order as in the test animals.

Group III:- Auto-transfusion with labelled in-vitro specific polysaccharide-sensitized (maximally and minimally) erythrocytes:

About half the total number of the animals in this group were primed with 2 mg. of polysaccharide each. They were left for 7 days and then auto-transfused with labelled sensitized cells.

The/

The other remaining animals in this group were also injected with labelled sensitized cells, but these were not primed.

Method for in-vitro Sensitization and ^{51}Cr labelling: Erythrocytes were collected, washed twice in sterile saline and suspended in saline at 10 per cent. The cells contained in these suspensions were then either maximally or minimally sensitized with S.gallinarum lipo-polysaccharide (Chapter IV). They were then washed 3 times in saline and labelled with appropriate amounts of ^{51}Cr . After re-suspension in autologous plasma-saline — except for injection into primed chickens — appropriate amounts of these cells were transfused. (Specific polysaccharide sensitization was verified each time after ^{51}Cr labelling by haemagglutination with the homologous antiserum).

These experiments, analogous to survival studies of ^{51}Cr labelled-sensitized erythrocytes in immune and non-immune animals, were controlled by a series of experiments involving the auto-transfusion of labelled, sham-sensitized cells into primed and normal chickens.

Group IV:- The effect and influence of passively administered specific, homologous antibody on the survival rates and pattern of elimination of in-vitro sensitized autologous erythrocytes:

There are two main advantages in carrying out these experiments:

(1) Known amounts of antibody can be administered so that the effects of low concentrations, below that which could be detected serologically, can be investigated.

(2) Known amounts of sensitized, labelled cells can be studied in relation to amounts of antibody present in circulation, and immune clearance rates can therefore be easily compared. This group of/

of animals was divided into 5 sub-groups, and various experiments, involving auto-transfusion of accurately measured doses of sensitized labelled cells, followed later by injection of various amounts of homologous antiserum, were performed. Survival curves of such studies were followed as indicated below:

(a) Effect of a fixed amount of homologous antiserum on various doses of maximally-sensitized cells.

(b) Effect of various amounts of homologous antiserum on a fixed dose of maximally sensitized cells.

(c) Effect of various amounts of homologous antiserum on a fixed dose of minimally sensitized cells.

(d) Effect of a second injection of homologous antiserum.

(e) Control experiments included animals receiving the same range of doses of sensitized, labelled cells, but sterile saline was injected in place of homologous antiserum. Other control chickens received sham-sensitized, labelled cells of the same dose range and the same amounts of homologous antibody as employed in the test animals.

In this group, accurately measured volumes of packed sensitized cells were injected without being suspended in autologous plasma-saline. However, the last traces of cells left in the syringe after injection were taken up in a small volume of plasma-saline and re-transfused. The whole injection exercise took less than 2 minutes.

Activity in these experiments was determined over a period of about 2 hours, taking the 10-minute sample as the 100 per cent survival. Samples were thus taken after 10, 30, 60, 120 /

120 and 150 minutes; and after injection of antibody, at 10, 30, 60, 120 minutes; 4, 6, 24 and 48 hours.

(2) Infection Experiments:

These consisted of all the survival studies in chickens which were infected with S.gallinarum. Adult 'Hybrid 66' hens were used, and these were divided into 2 groups:

Group 1:- Erythrocyte Survival Rates in Infected Chicken: Some of these animals were infected 24 hours after they have been transfused with labelled autologous erythrocytes. Another batch of chickens were infected 7 days after daily elimination of auto-transfused cells had been monitored.

Control studies consisted of injection of sterile broth culture, instead of viable S.gallinarum.

In these experiments, the initial radioactivity injected was doubled by labelling the cells from 10 mls. of blood withdrawn with a radiochromium solution containing an activity of 60 to 70 μ C.

Group 2:- Cross-transfusion Erythrocyte Survival Studies: A number of the animals were homo-transfused with either Type II or III in-vivo sensitized, labelled erythrocytes which had previously been checked for sterility (Chapter IV). These cells were injected suspended in sterile saline, and in contrast to the measurements involving infected chickens, activity was expressed as counts per second per unit haematocrit since the total red cell mass remained stable throughout the measurements (Veall and Vetter, 1958; Blahd, 1962).

Some of the animals in this group were also injected with homologous, non-sensitized (normal) labelled erythrocytes at various/

various stages during infection with fowl typhoid. Thus, the animals were transfused either 2, 3, 4 or 5 days after being infected.

Control experiments in this group consisted of injection of normal, labelled homologous cells, taken from the same donor chickens which were subsequently infected and their in-vivo sensitized cells utilised as described above. Other animals received labelled cells at various times after injection of a sterile broth digest culture.

Terminology: It was considered important to define some of the terms used.

(a) Where the term 'haemolysis' is used it will denote an increased rate of erythrocyte destruction; it will not imply that this is due to any particular mechanism, or that haemolysis is intravascular with haemoglobinaemia.

(b) 'Overt haemolysis' will also denote clinically obvious haemolysis as shown by anaemia, bilirubinaemia and gross reticulocytosis.

(c) 'Latent or Occult haemolysis' will denote haemolysis which is not clinically obvious, but which can be demonstrated by pigment excretion and/or erythrocyte survival studies.

(d) Haemolytic anaemia is equated with overt haemolysis; excessive haemolysis means a rate of erythrocyte destruction which is greater than normal, and which may or may not be clinically obvious.

RESULTS

(1) Erythrocyte Survival in Normal Chickens:

The normal plot of ^{51}Cr labelled autologous erythrocyte/

erythrocyte disappearance, over 30 days is a slightly curvilinear slope which is not truly exponential (Fig. 32). Assuming a constant rate of elution of ^{51}Cr the slope of this line is then an accurate reflection of the erythrocyte life-span (Mollison, 1967). As an index of the rate of ^{51}Cr disappearance, the time for 50 per cent of the isotope to disappear from the circulation is usually taken. This is the half life, and was found to be between 13 and 14 days in the chicken. These determinations compare well with the recent finding of half-life of over 13 days in chickens by Wels et al., (1967).

The normal mean cell-life (MCL) and the maximum cell-life were also calculated, according to Dornhorst's method (Dornhorst, 1951), to be 21 and 33 days respectively. Maximum life span of chicken erythrocytes had previously been estimated by the radiochromium method to be 30 days (Rodnan et al., 1957) and a range of figures between 28 and 32 days had also been obtained by Hevesy and Ottesen (1945) and Ottesen (1948) using other techniques.

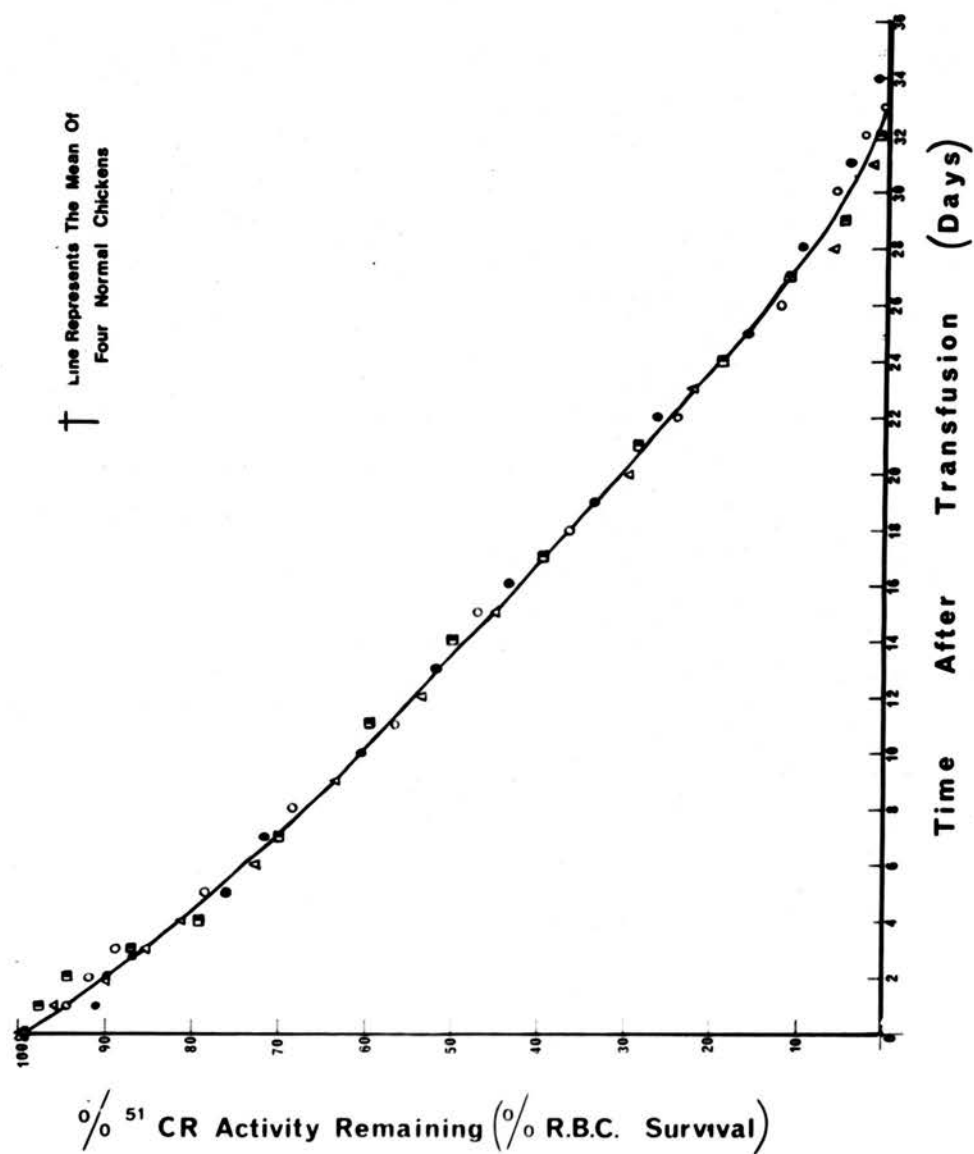
Unlike mammalian erythrocytes, it has been found that the in-vivo elution of ^{51}Cr does not appreciably influence the estimation of the maximum life-span of avian erythrocytes (Rodnan et al., 1957).

(2) Erythrocyte Survival in Infected Chickens:

These studies describe experiments with ^{51}Cr labelled erythrocytes in chickens infected with virulent S.gallinarum and normal chickens homo-transfused with erythrocytes which had been previously sensitized in-vivo during infection.

(i) Auto-survival of Erythrocytes: Survival curves obtained for
a /

FIGURE 32. NORMAL ERYTHROCYTE SURVIVAL CURVES



a group of infected and normal chickens are shown in Fig.33. The half-life of labelled autologous cells was very significantly shortened as compared with control animals: from 3.25 to 4.75 days, as opposed to 12.5 to 14.5 days respectively (Table 19). This Table also shows that the maximum cell life-span of the infected animal was also drastically reduced — from a mean value of over 30 days to between 5 and 6.5 days. Animals which were infected, either 24 hours or 7 days after auto-transfusion of labelled cells, all characteristically showed an acceleration of erythrocyte loss a few hours before death, which was between the fourth and sixth days after infection.

The surviving residual blood radioactivity depended on the time of death of the chicken. For example, chicken number 202, which died within 4 days after infection, still had nearly 34 per cent of the blood radioactivity remaining. On the other hand, there was less than 13 per cent of total blood radioactivity in chicken number 613 which died after 6 days. Measurements of plasma radioactivity showed no significant deviation from background activity to indicate release of ^{51}Cr . Furthermore, there was no increase in plasma haemoglobin.

(ii) Cross-Transfusion Studies in Infected Chickens: Having established that erythrocytes are rapidly destroyed during fowl typhoid the next step was to examine the behaviour of normal homologous erythrocytes in infected animals, and conversely, of in-vivo erythrocytes in normal chickens. Such studies are important in the demonstration of the factors responsible for the initiation of the haemolytic process.

(a)/

FIGURE 33.

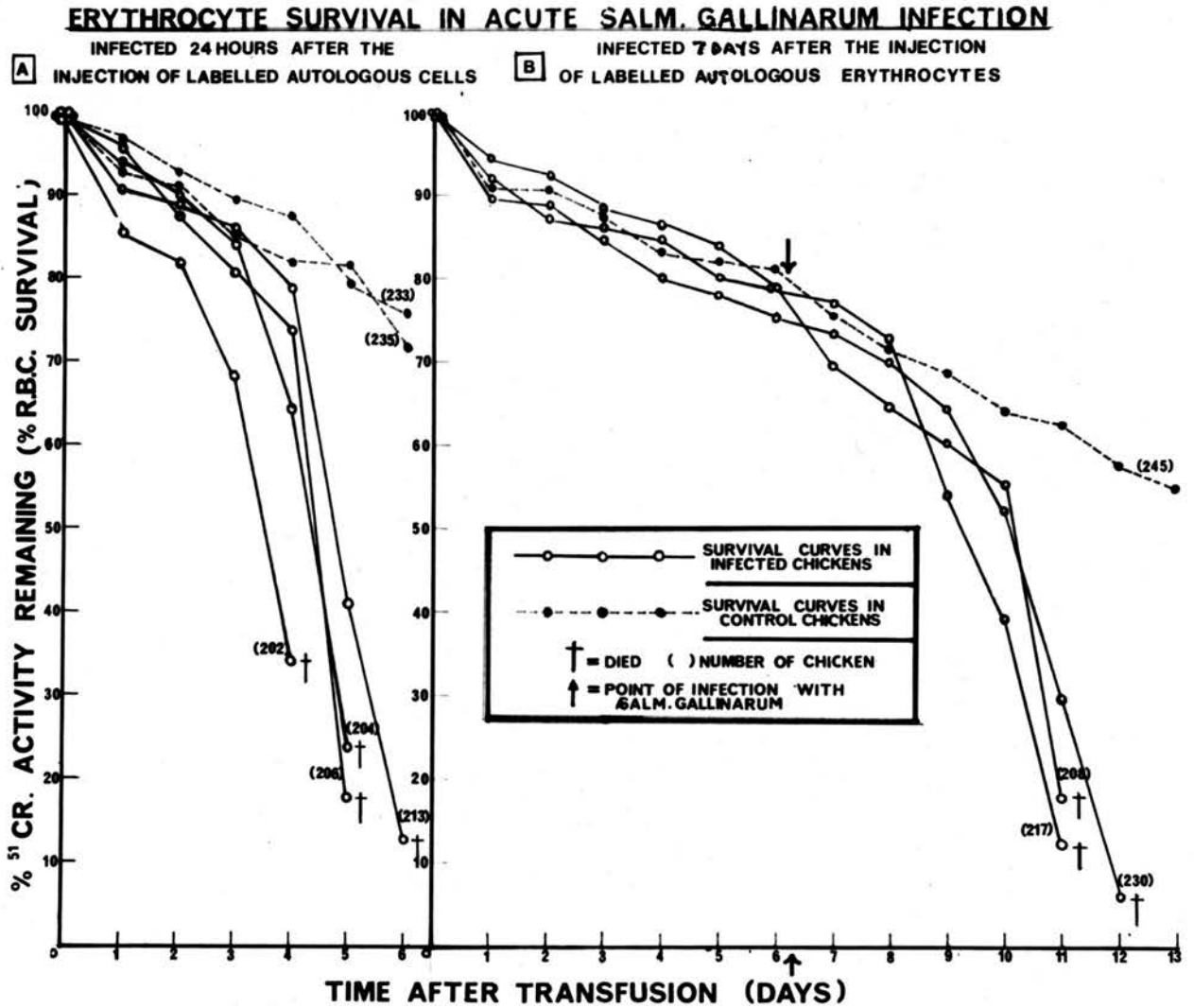


TABLE 19

ERYTHROCYTE AUTO-SURVIVAL AND ANAEMIA (HÄMATOCRIT CHANGE) IN CHICKENS WITH FATAL FOWL TYPHOID

GROUP	CHICKEN NO: (A/-)	P.C.V. (%)	TIME OF DEATH AFTER INFECTION (DAYS)	ERYTHROCYTE HALF-LIFE ($t_{1/2}$ -CR) (DAYS)	ERYTHROCYTE MAXIMUM CELL LIFE (DAYS)	PERCENTAGE RESIDUAL BLOOD RADIOACTIVITY AT TIME OF DEATH
INFECTED	206	18.0	5	4.5	5.25	17.9
	202	21.0	4	3.5	5.0	33.8
	213	16.5	6	4.75	6.5	12.7
	204	20.5	5	4.5	5.75	24.1
	208	19.0	5	4.25	5.5	18.4
	230	17.0	6	4.0	6.25	6.2
	217	18.5	5	3.25	5.5	12.6
CONTROL	233	36.0		13.0	32.0	
	235	35.0		14.5	30.5	
	245	33.5		12.5	29.5	

(a) Survival of Normal Erythrocytes in Infected Chickens: The results of survival of normal, homologous erythrocytes in acutely infected chickens are depicted in Fig. 34, which shows that normal erythrocytes are destroyed rapidly in chickens infected with S. gallinarum. It could not be ascertained whether these cells became sensitized after being transferred to their new host before being eliminated. However, it is quite conceivable that cells which were transfused 2 or 3 days before death were subsequently sensitized before destruction. This suggestion is substantiated by the finding that chicken (for example, number 254) transfused with erythrocytes less than 24 hours before death showed only slight decrease in erythrocyte survival. In the latter animal, there was still well over 65 per cent radioactivity present in the circulation at the time of death. In contrast, chickens (numbers 210, 223 and 224) which were transfused with normal erythrocytes more than 3 days before death, exhibited very marked decreases in concentration of radioactivity, and, on the average, only slightly more than 10 per cent blood radioactivity remained at the time of death.

(b) Survival of *in-vivo* sensitized Erythrocytes in Normal Chickens: When either in-vivo Type II or III sensitized, labelled cells were transfused into normal chickens, a proportion of these cells were rapidly destroyed (Fig. 35). This followed from the survival curves which appeared to consist of 2 components or cell populations: an initial steep slope followed by a much less steeply falling slope. Using the formula given by Dacie and Lewis (1968) it was found that over 70 per cent of the injected labelled cells were rapidly destroyed, with a mean half-life of 4 days, and a maximum life-span of/

FIGURE 34. SURVIVAL OF NORMAL ERYTHROCYTES IN INFECTED CHICKENS

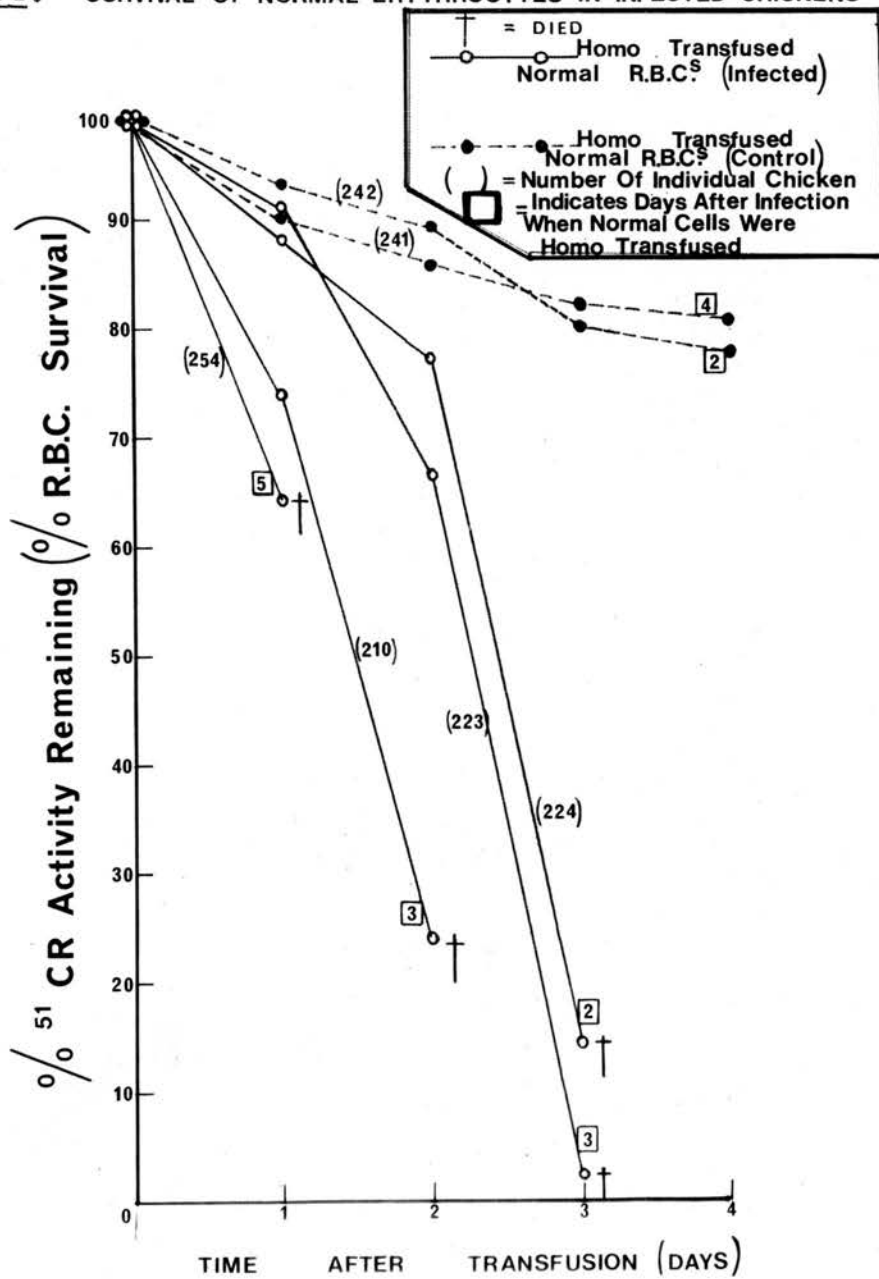
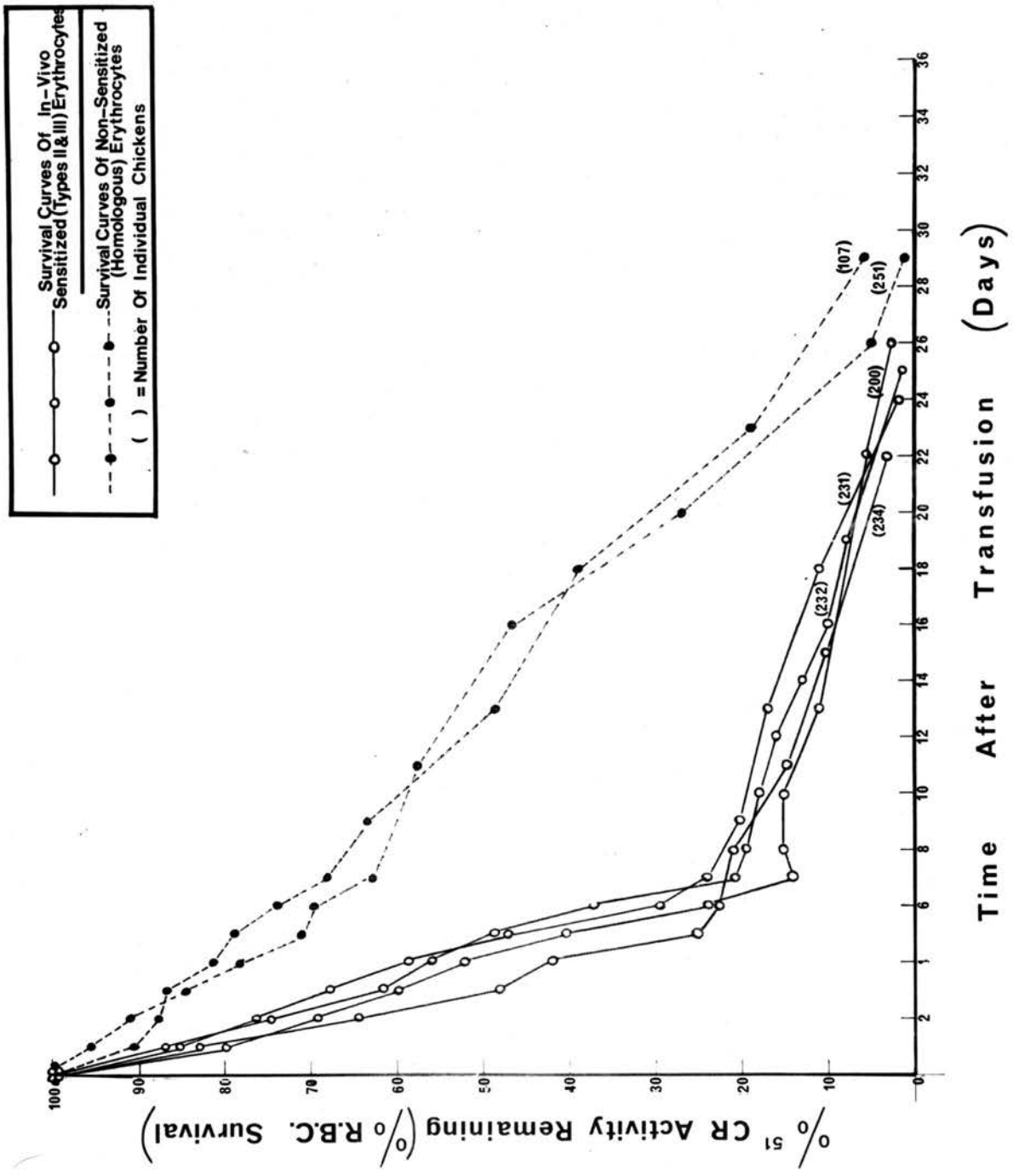


FIGURE 35. SURVIVAL OF IN-VIVO SENSITIZED ERYTHROCYTES IN NORMAL CHICKENS



of 6 days. The life-span of the remaining 30 per cent radioactivity was also found to be 28 days, and the MCL of the entire cell population was deduced to be 8 days.

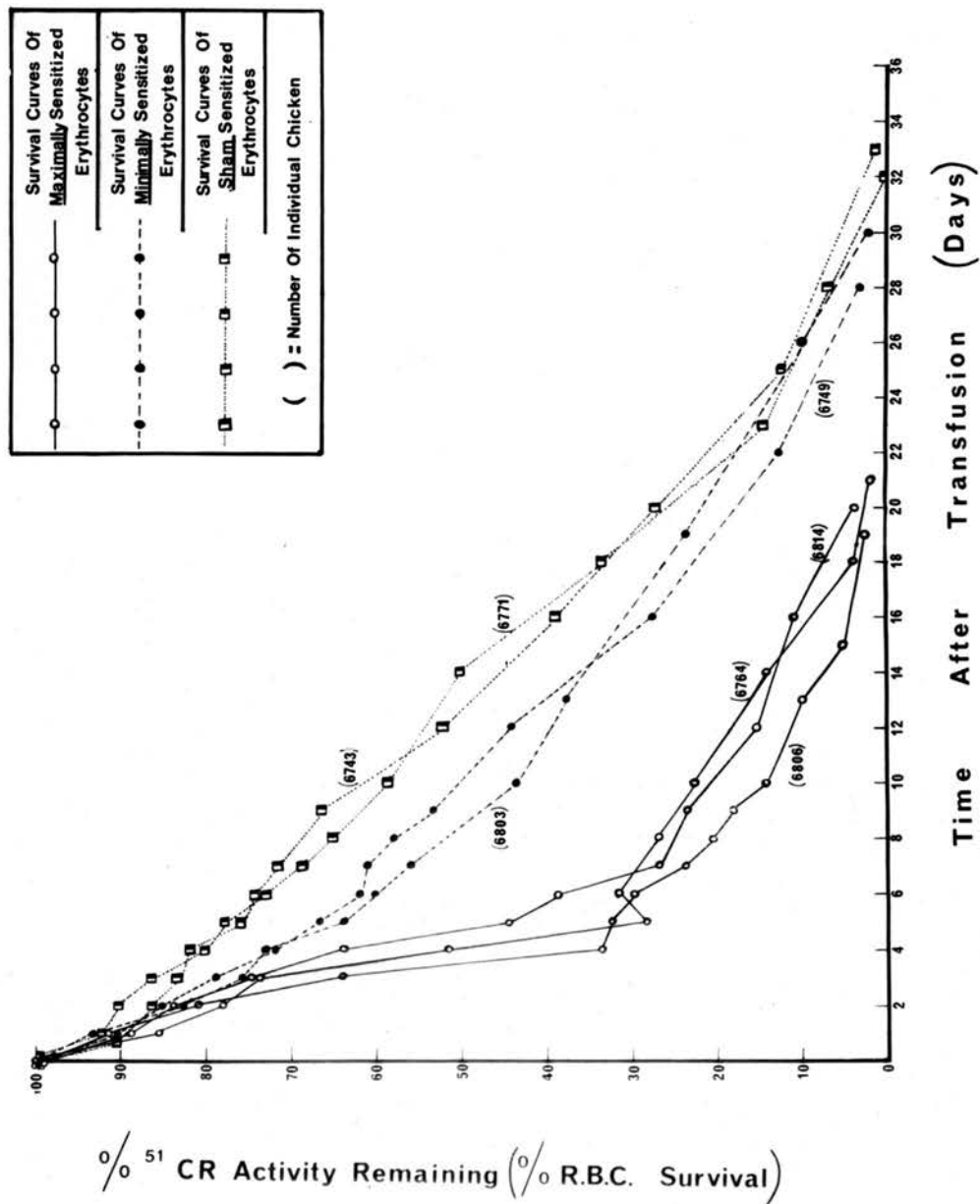
(3) Survival of Erythrocytes Sensitized with *S.gallinarum* LPS in Primed (Immune) and Non-immune Chickens:

To investigate the role of immune mechanisms in mediating the clearance of sensitized erythrocytes, these studies were undertaken to measure the survival of erythrocytes that had been sensitized in-vitro with *S.gallinarum* polysaccharide in non-immune and immune (primed) chickens. The results of this investigation are illustrated in Figs.36 and 37.

Erythrocytes maximally sensitized and transfused into normal, non-immune chickens had appreciably shortened survival as compared to normal erythrocytes, with a range of half-lives between 3.5 and 4.5 days and mean of maximum cell life of 23.5 days (Fig.36). This figure also shows that the individual survival curves could be resolved into 2 components: an initial steep slope followed, at approximately 5 days, by a change to a more gradual decline. It is noteworthy that all the non-immune animals eventually produced specific LPS-antibodies as a result of being challenged with maximally sensitized cells. The haemagglutinating titres, 3 days after injections, ranged from 1:20 to 1:80, and by the fourteenth day they had risen further and ranged from 1:1280 to 1:5120. However, this high titre did not seem to affect the clearance rate as after the fifth or sixth day as already described, increased survival became apparent.

In contrast, in chickens primed with LPS and auto-transfused/

FIGURE 36. SURVIVAL OF SENSITIZED ERYTHROCYTES IN (Non-Primed) CHICKENS



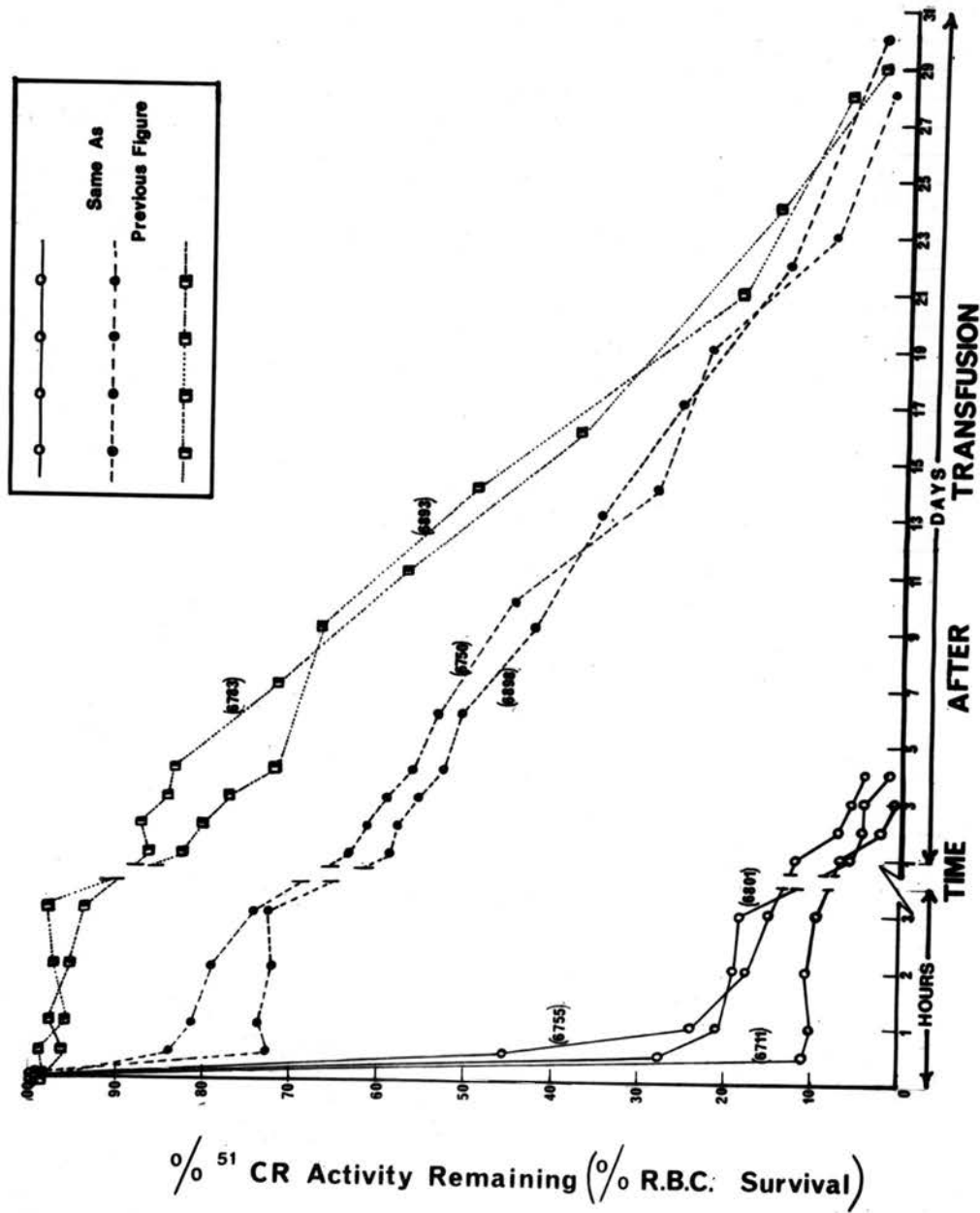
transfused with maximally sensitized cells 7 days afterwards, striking destruction occurred within 15 to 30 minutes after transfusion (Fig.37). Because of this marked initial haemolysis, the labelled erythrocytes did not survive beyond the first 24 hours. Subsequent surviving radioactivity was found to be mainly present in the plasma, as evidenced by the slight haemoglobinaemia and increased plasma radioactivity. The initial 50 per cent loss in the animals ranged from 15 to 30 minutes, and all the sensitized, labelled cells had been eliminated within 90 minutes after transfusion.

It was also of interest to record that the priming of chickens with LPS, prior to transfusion with sensitized cells, elicited the production of specific haemagglutinating titres ranging from 1:256 to 1:1024, approximately 7 days after the injections.

Quite different results were obtained when erythrocytes were minimally sensitized and auto-transfused into normal and primed chickens. In non-immune animals, an appreciable amount of radioactivity was lost within 24 hours, but the subsequent survival of the cells showed only a slight deviation from the normal range (Fig.36). The half-lives ranged from 8.5 to 10 days, and the cells survived in the circulation on an average of about 30 days. Two-component curves were not observed as with the maximally sensitized cells, and the survival curves showed reasonably smooth, but slight curvilinearity.

It was, however, surprising to find that only a slight deviation from the pattern of cell destruction, as above, was obtained when minimally sensitized cells were transfused into primed (immune) chickens./

FIGURE 37. SURVIVAL OF SENSITIZED ERYTHROCYTES IN (primed) CHICKENS



chickens. The only difference was the exaggerated, rapid, initial clearance observed soon after transfusion (Fig.37). Survival after 24 hours was not markedly different from minimally sensitized cells transfused into non-immune chickens, and the half- and maximum cell lives were determined respectively to be between 6.5 and 8.5 days and 29 and 32 days.

It was also of interest to note that cells, even moderately altered by polysaccharide, showed appreciable shortening of their half-lives in immune animals as compared with the values obtained for the sham sensitized cells, which were between 13 and 14 days (Fig.37). However, the half-life range of 6.5 to 8.5 days for the minimally sensitized cells in immune animals was still significantly higher than even the range obtained after injection of maximally sensitized cells into normal, non-immune chickens (3.5 to 4.5 days). These results reveal that erythrocytes sensitized in-vitro with high concentrations of polysaccharide are rapidly eliminated from the circulation of both immunized and non-immunized chickens. However, the presence of homologous antibody ensures nearly a 200-fold decrease in the half-life of maximally sensitized erythrocytes as compared with the survival time of the same type of cells in the absence of specific antibody (Figs.36 and 37). This dramatic effect of antibody on the rate of clearance of sensitized cells was, however, not observed with erythrocytes sensitized with minimal amounts of bacterial polysaccharide. These minimally sensitized cells were found to be cleared at nearly the same rate both in the presence and absence of homologous bacterial antibodies.

(4) Effect of Intravenously Injected Large Dose of Endotoxin on Erythrocyte Auto-survival:

It/

It was of interest to determine whether endotoxin (LPS) injected directly into the circulation alters the survival of labelled erythrocytes. Accordingly, experiments were carried out to show the effect of injection of a large sensitizing dose of LPS (40 mgm. per Kgm.). The results of such survival studies are depicted in Fig. 38. Chickens which received these large doses of endotoxin exhibited the following extremely interesting characteristic survival curves:

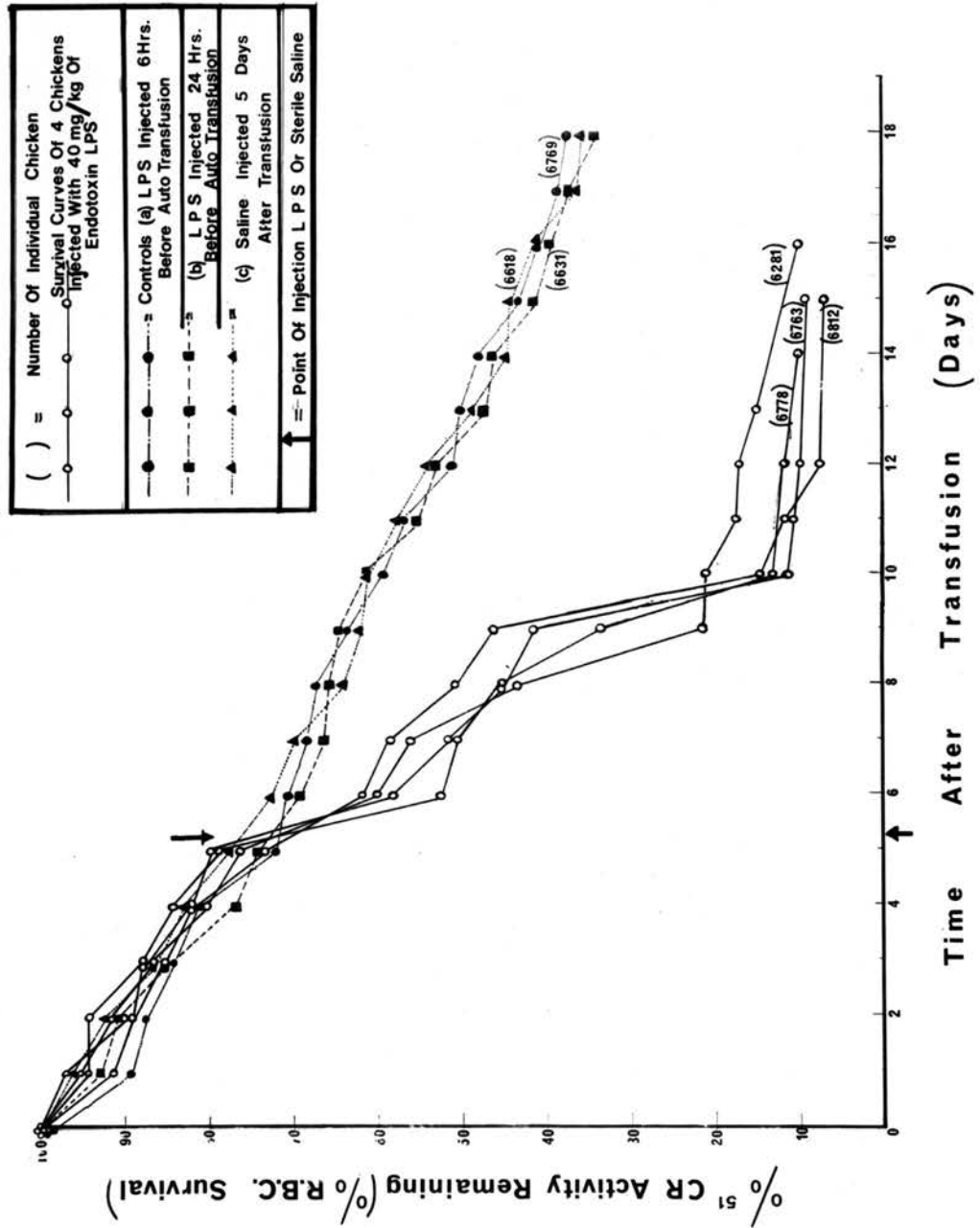
(i) There was an immediate increase in the rate of disappearance of radioactivity after the injection of the LPS. In addition, there were daily decreases in erythrocyte survival which were, however, small but uniform.

(ii) However, 4 or 5 days after the injection, a sudden and rapid loss of a large amount of radioactivity occurred, and the final outcome of such a loss of labelled erythrocytes was the development of a moderate to severe clinical anaemia.

It was found that, on average, the mean haematocrit was reduced from an initial value of 32 to approximately 20 per cent at the peak of anaemia. (All the chickens survived this haemolytic crisis, and recovered fairly rapidly. For instance, by the tenth day after LPS injection nearly all the animals had returned to normal. Centrifugation of a blood sample, taken at this point, showed that the residual radioactivity was almost exclusively in the plasma.

Control chickens which received the LPS either 6 or 24 hours before the transfusion of labelled erythrocytes exhibited a greater decrease in concentration of radioactivity in the first 24 hours than did animals which received sterile saline, followed/

FIGURE 38. EFFECT OF LARGE SINGLE DOSE OF ENDOTOXIN ON
ERYTHROCYTE SURVIVAL



followed by labelled erythrocytes. However, the half-life of the cells after 24 hours was within normal range in both control groups (12.5 to 13.5 days) (Fig.38).

(5) Effects of Passively Administered Antibody on Clearance of Sensitized Erythrocytes:

In view of the indications that antibody was concerned in the enhanced clearance of sensitized erythrocytes, radiochromium experiments were designed to study the action of such antibodies on phagocytosis of autologous erythrocytes carrying the specific salmonella antigen.

(a) Rate of Clearance of Sensitized Autologous Erythrocytes:

The results of these experiments, summarized in Table 20 and illustrated in Fig.39 (A, B and C), show that the rate of blood clearance of sensitized erythrocytes was greatly increased by the injection of the specific anti-salmonella LPS-serum as indicated by the range of half-lives of treated and untreated control chickens.

The rate of blood clearance of 50 per cent of maximally sensitized cells was significantly greater than minimally sensitized cells (Table 20.) Depending on the amount of injected specific antibody, the half-life of maximally sensitized cells ranged from 7.5 to 27 minutes; corresponding figures for minimally sensitized cells were from 1.5 to 9 days. When sterile saline was injected in place of specific antibody, the half-life of maximally sensitized cells was still markedly reduced (that is, between 4 and 5 days). On the other hand, sham sensitized cells survived normally whether sterile saline or homologous antibody was injected (12.5 to 13.5 days).

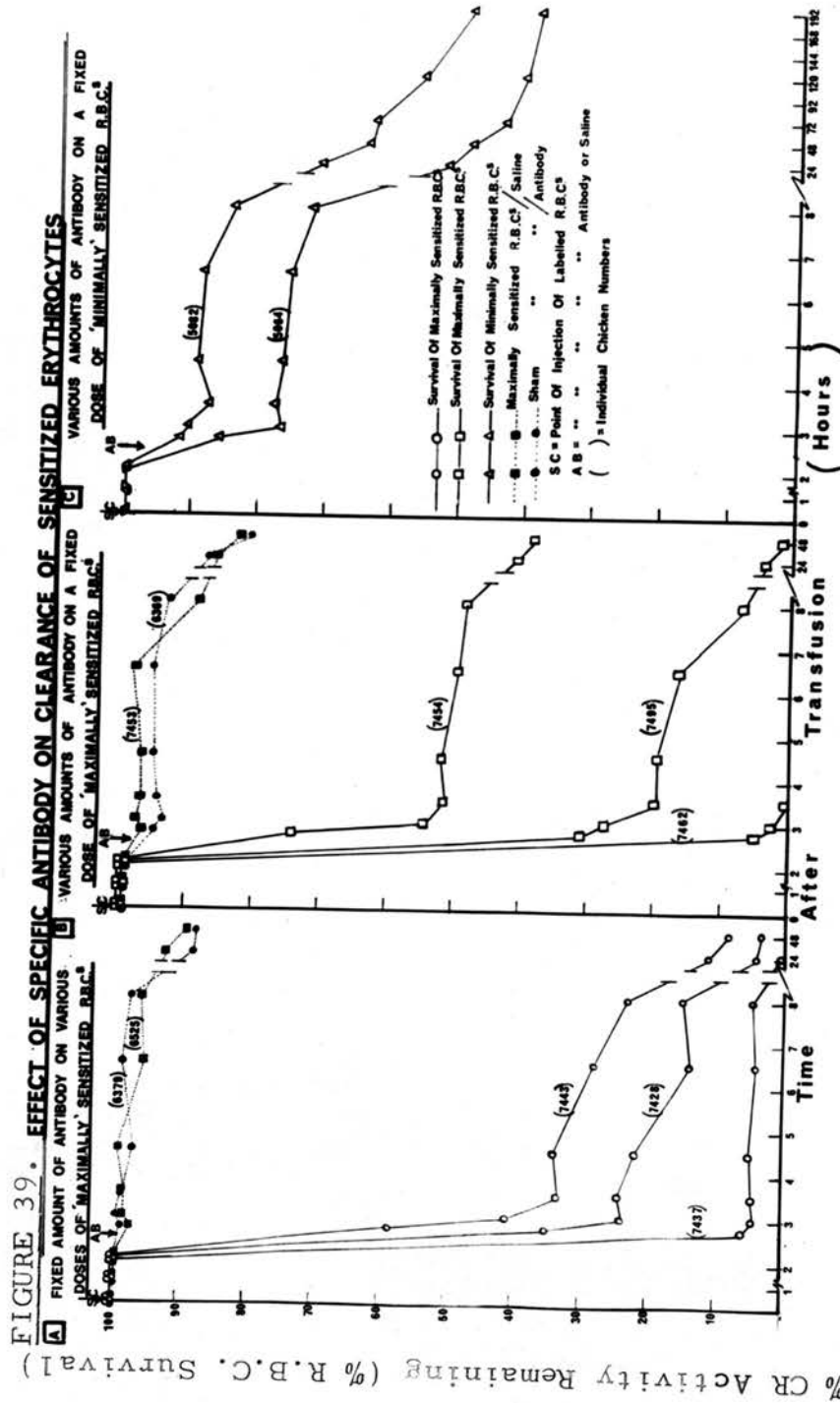


TABLE 20

RATE OF CLEARANCE OF SENSITIZED ERYTHROCYTES IN CHICKENS INJECTED WITH SPECIFIC ANTI-O SERUM

GROUP	CHICKEN NO. (68/-)	DOSE OF SPECIFIC ANTIBODY * (ml/Kgm)	TIME TO CLEAR 50 PER CENT OF ERYTHROCYTES [†]					
			'MAXIMALLY' SENSITIZED			'MINIMALLY' SENSITIZED	'SHAM' SENSITIZED	
			0.1 ml/Kg	0.5 ml/Kg	1.0 ml/Kg		0.5 ml/Kg	1.0 ml/Kg
TREATED	7437	2	7.5 MINS.	-	-	-	-	-
	7428	2	-	10.5 MINS.	-	-	-	-
	7443	2	-	-	18.0 MINS.	-	-	-
	7454	1	-	27.0 MINS	-	-	-	-
	7495	2.5	-	12.0 MINS.	-	-	-	-
	7462	5	-	10.5 MINS.	-	-	-	-
	5064	5	-	-	-	1.5 DAYS	-	-
	5082	1	-	-	-	9.3 DAYS	-	-
	6525	2 (SS)	-	5 DAYS	-	-	-	-
	6379	2	-	-	-	-	13.5 DAYS	-
CONTROLS	6369	5	-	-	-	-	-	12.5 DAYS
	7453	5 (SS)	-	-	4 DAYS	-	-	-

SS = STERILE SALINE

* = ⁵¹CR HALF-LIFE

† = FINAL HEMAGGLUTININATING TITRE OF 1/5120

Table 20 and Fig. 39B also show the rates of clearance of a moderate fixed dose (0.5 ml. per Kgm.) of maximally, minimally and sham sensitized erythrocytes, related to various amounts of homologous antibody to be injected. With maximally sensitized cells, the fastest rates of clearance were observed in animals receiving the largest amount of antibody and the slowest rates in those receiving the smallest amount of antibody. For example, a half-life of 10.5 minutes was obtained when chicken number 7462 was given 5 mls. of antibody per Kgm. whilst chicken number 7454, which received only 1 ml. of antibody per Kgm., gave an erythrocyte survival half-life of 27 minutes. However, the correlation in the intermediate cases, between the dose of antibody and the rate of clearance, was not close. It was observed that the rate of clearance of maximally sensitized cells was always exponential, even when clearance was slow (Fig. 39A and B). There was also evidence that the curves had more than one component after the radioactivity measurements were plotted semi-logarithmically.

In contrast, minimally sensitized cells auto-transfused into normal chickens (numbers 5064 and 5082) showed half-lives of 1.5 and 9.3 days respectively when challenged later with 5 mls. and 1 ml. per Kgm. of homologous antibody respectively (Table 20, Fig. 39C).

- (b) Plasma, Radioactivity after Clearance: Determination of plasma radioactivity in representative animals from this group indicated that destruction of sensitized cells was extravascular, rather than intravascular. Thus, chicken numbers 7454 and 7433, in which clearance was relatively slow, the plasma radioactivity at the conclusion of the experiments was less than the equivalent of 2 per cent of the total injected dose. However, in cases in which the clearance/

clearance was very rapid the amount of plasma radioactivity reached values equivalent to 5 or 6 per cent of the total injected dose. For example, in chicken number 7428, in which the cells were cleared with a half-life of 10.5 minutes, the plasma radioactivity at 30 minutes — after more than 75 per cent of the cells had been cleared from the circulation — was 5.8 per cent, and at 5.5 hours it was only slightly over 6 per cent. Similarly, in chicken number 7437, one hour after injection of specific antibody — at a time when 95 per cent of the cells had been eliminated — the amount of plasma radioactivity was not more than 5 per cent of the total injected dose.

These small but gradual and slow increases in plasma radioactivity indicated that this activity entered the circulation slowly, and probably after the phagocytosis and destruction of the labelled erythrocytes by the cells of the reticuloendothelial system, rather than by direct liberation from damaged erythrocytes.

(c) Clearance of Small and Large Doses of Sensitized Erythrocytes:

Survival of various doses of labelled, maximally sensitized cells was estimated in a group of chickens, and some representative results are given in Table 20 and Fig.39A. Fig.39A shows that in the animal (number 7437) receiving only 0.1 ml. of sensitized cells per Kgm., clearance was approximately exponential and very rapid (the half-life was 7.5 minutes), and it involved virtually over 95 per cent of the injected cells. On the other hand, in the animal number 7443, receiving a larger dose of sensitized cells (1 ml. per Kgm.), a two-component curve was observed: 55 per cent of the cells being cleared relatively rapidly (half-life was 18 minutes),/

minutes), and the remainder very slowly, with a mean cell life of about 5 days. The patterns of clearance were identical in the animal (number 7428) which received a moderate dose of sensitized cells (0.5 ml. per Kgm.) and the one (number 7495) which was injected with an intermediate amount of homologous antibody (2.5 ml. per Kgm.) (Fig.39B). All other chickens given various doses of sensitized cells were injected with a fixed standard amount (2 mls. per Kgm.) of homologous antibody.

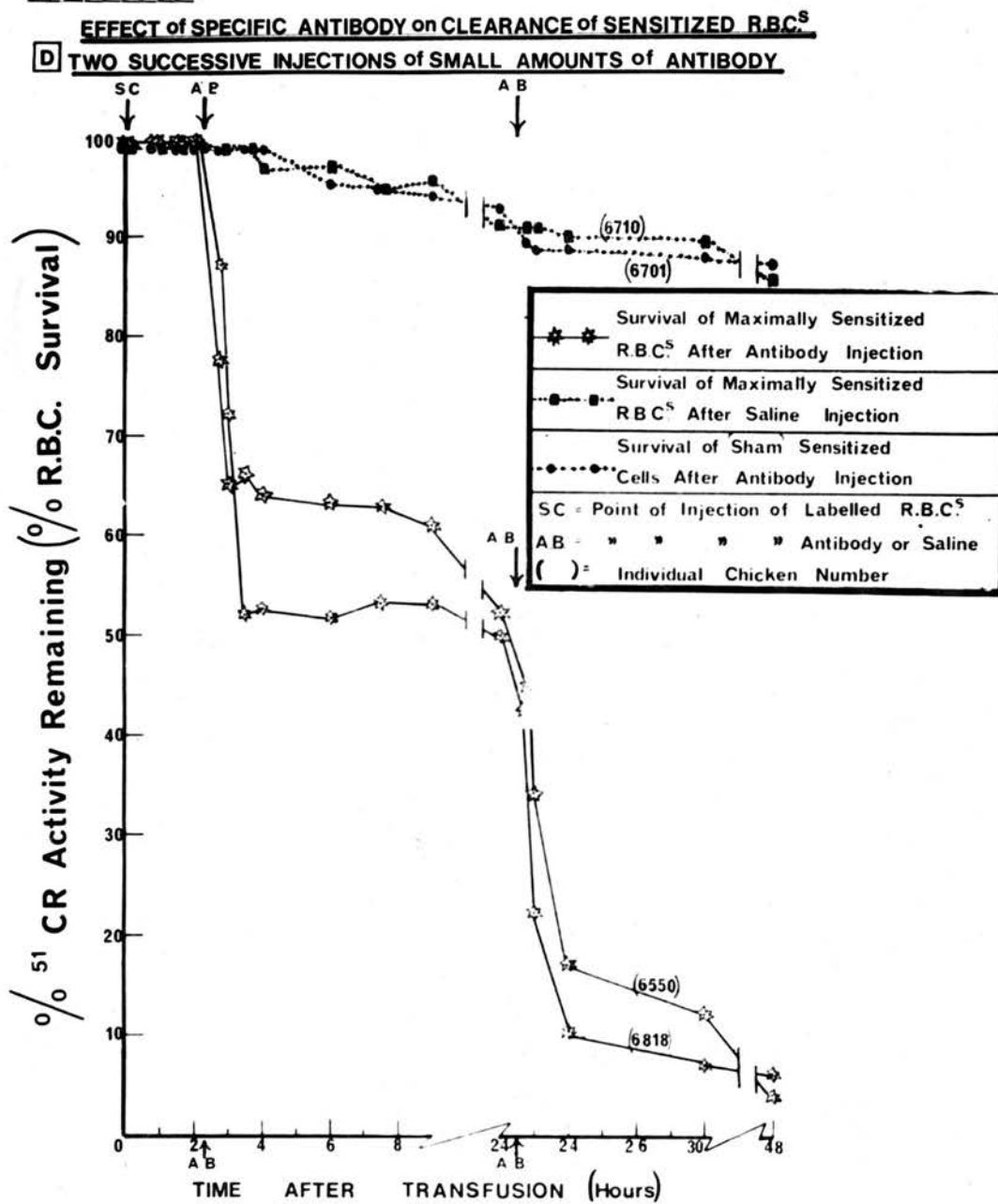
It was also observed that in animals in which there was only partial clearance in the first 1-2 hours, cells surviving at the end of that time were only slowly eliminated. For example, in chicken number 7454 (Fig.39B), where cell survival after 24 hours was 40 per cent, survival at 48 hours was still 38.5 per cent.

(d) Clearance after Two Successive Injections of Antibody: Evidence was sought to support the hypothesis that one of the causes of the two-component curves obtained above may be the 'exhaustion' of antibody, as well as to uneven sensitization of the erythrocyte population; Fig.40 illustrated such studies.

Chicken numbers 6550 and 6818 were given 2 mls of maximally sensitized cells per Kgm., followed 24 hours later by the injection of 1.5 ml. of homologous antibody per Kgm. After a 'plateau' had been reached, a second injection of the same amount of antibody was given intravenously, causing prompt destruction of over 80 per cent of the previously surviving cells.

In one case, not recorded, the second dose of antibody was delayed until 3 days after the cells had been injected, and these were again rapidly cleared. This clearly demonstrated that there is no question of elution of polysaccharide from the cells; furthermore, this/

FIGURE 40.



this finding reinforces the conclusion that rapid clearance of sensitized cells was mediated through the action of the specific antibody.

(6) Sites of Erythrocyte Destruction:

Full understanding of the disappearance of radioactivity requires quantitative study of the rate and route of excretion, as well as the sites of isotope deposition. The results of such studies, providing direct evidence of the pattern of erythrocyte destruction, are summarized in Table 21. This shows clearly that the particular sites of erythrocyte destruction depend on the degree of sensitization and the rate of elimination. This relationship is also indicated by the ratio of the spleen to liver activity.

Organ radioactivity distribution was studied by determining the amounts of ^{51}Cr activity in spleen and liver at the conclusion of the various experiments. As Table 21 shows, residual radioactivity distribution in these organs in the infected chickens was very different from the pattern observed in control chickens. On the average, over 70 per cent of the total injected radioactivity was present in the spleen and liver combined. Each of these organs destroyed approximately equal amounts of labelled erythrocytes. However, in terms of unit organ wet weight, the spleen is considerably more active.

Normal chickens (for example, numbers 6764 and 6806), which were auto-transfused with in-vitro maximally sensitized cells, also showed proportionately higher splenic cell destruction than the liver, and the spleen/liver activity ratios, as compared with control/

TABLE 21

DISTRIBUTION OF ORGAN RADIOACTIVITY IN CHICKENS AFTER VARIOUS TREATMENTS

EXPERIMENTAL GROUP	CHICKEN NUMBER (68/-)	TOTAL INITIAL INJECTED RADIOACTIVITY (COUNTS/MIN.)	TOTAL ORGAN RADIOACTIVITY (COUNTS/MIN.)		% ORGAN ⁵¹ CR ACTIVITY OF TOTAL INJECTED DOSE	SPLEEN ACTIVITY LIVER RATIO (ORGAN ⁵¹ CR IN ONE GRAM OF WET TISSUE)
			SPLEEN	LIVER		
'MAXIMALLY' SENSITIZED CELLS AUTO- TRANSFUSED INTO NORMAL (NON-IMMUNE) CHICKENS	6764	400.0	136.4	113.6	34.1	38.4
	6806	466.7	193.7	102.7	41.5	22.0
'MINIMALLY' SENSITIZED CELLS AUTO- TRANSFUSED INTO NORMAL (NON-IMMUNE) CHICKENS	6749	416.7	253.4	47.1	60.8	11.3
	6803	433.3	214.5	71.9	49.5	16.6
'SHAM' SENSITIZED CELLS AUTO-TRANSFUSED INTO NORMAL (NON-IMMUNE) CHICKENS	6771	400.0	216.8	33.6	54.2	8.4
	6734	366.7	177.8	48.0	48.5	13.1
'MAXIMALLY' SENSITIZED CELLS AUTO- TRANSFUSED INTO PRIMED (IMMUNE) CHICKENS	6801	400.0	29.2	258.4	7.3	64.6
	6711	416.7	20.4	295.0	4.9	70.8
'MINIMALLY' SENSITIZED CELLS AUTO- TRANSFUSED INTO PRIMED (IMMUNE) CHICKENS	6750	350.0	207.2	69.7	59.2	19.9
	6898	383.3	205.8	59.0	53.7	15.4
'SHAM' SENSITIZED CELLS AUTO-TRANSFUSED INTO PRIMED (IMMUNE) CHICKENS	6783	433.3	192.4	102.3	44.4	23.6
	6893	366.7	195.1	43.6	53.2	11.9
INJECTION OF SPECIFIC ANTISERUM AFTER AUTO-TRANSFUSION OF :	7428	133.3	7.2	104.9	5.4	78.7
(i) 'MAXIMALLY' SENSITIZED ERYTHROCYTES:	7462	187.5	4.1	161.4	2.2	86.1
(ii) 'MINIMALLY' SENSITIZED ERYTHROCYTES:	5082	141.7	38.0	13.3	26.8	9.4
	5064	187.5	64.9	22.9	36.6	12.2
(iii) 'SHAM' SENSITIZED ERYTHROCYTES:	7453	200.0	16.8	3.4	8.4	1.7
	6379	237.5	14.5	5.9	6.1	2.5
INFECTED WITH ACUTE FOWL TYPHOID:	(A/-)					
	206	1333.3	456.0	433.3	34.2	32.5
	213	1133.3	290.1	585.9	25.6	51.7
	208	1266.7	373.7	511.7	29.5	40.4
	230	1366.7	414.1	625.9	30.3	45.8
	233	1266.7	157.1	34.2	12.4	2.7
CONTROLS WITH STERILE BROTH CULTURE:	245	1200.0	223.2	46.8	18.6	3.9
						31.5

control animals given sham sensitized cells (found to be over 31), were respectively 7.2 and 11.6.

In the case of minimally sensitized cells transfused into immune chickens (for example, numbers 6750 and 6898) the results suggested that the spleen was more efficient in clearing these cells than the liver. The striking nature of this splenic activity was shown by the finding that, of the 70 per cent of total injected radioactivity recovered in the spleen and liver together, more than 50 per cent were deposited in the spleen. A similar effect was found when autologous, minimally sensitized cells were transfused into normal chickens, and specific antiserum was injected later (see chicken numbers 5082 and 5064 in Table 21).

In contrast to the above, greater liver deposition of activity was observed in cases where the cells were rapidly eliminated; that is, maximally sensitized cells in the presence of large quantities of specific antibody. This is exemplified by chicken numbers 6801 and 6711 where, of more than 70 per cent of the injected radioactivity recovered in the spleen and liver combined, more than 60 per cent were in the liver. Similar activity distribution was also observed in chicken numbers 7428 and 7462: of the approximately 90 per cent activity deposited in spleen and liver, the liver alone was found to contain 80 per cent of this activity.

These results therefore showed that erythrocytes, which were rapidly eliminated from the circulation — a direct function of both the quantities of adsorbed polysaccharide and homologous antibody — are sequestered mainly by the liver. Such cases, related to their spleen/liver activity ratios, were found to/

to have very low values. In contrast, cells which are slowly removed from the circulation are destroyed mainly in the spleen, and these have relatively higher spleen/liver activity ratios.

DISCUSSION

The results obtained here with ^{51}Cr labelled-erythrocytes, especially the finding of abnormal shortening of erythrocyte survival time during acute fowl typhoid, provide convincing direct evidence of increased erythrocyte destruction as probably the sole mechanism for the causation of the anaemia.

Until the present studies were carried out, it was not known which of the following mechanisms was responsible for the anaemia:

- (1) Haemolysis: either (a) pathological increase in the normal mechanism of effete erythrocyte removal, or (b) intravascular lysis of erythrocytes.
- (2) Inhibitory effect on blood forming tissues; that is, dyshaemopoiesis. From earlier observations (Chapters II and III) it is clear that both intravascular haemolysis and dyshaemopoiesis can be ruled out as significant factors. The present studies have, however, established unequivocally that the anaemia of acute fowl typhoid is haemolytic and the indications are that the mechanism responsible for its induction has an immunological basis.

The radiochromium-tracer method has been used extensively by several workers in the evaluation changes that occur spontaneously during the course of many types of haematologic diseases of man and animals (Veall and Vetter, 1958; Dacie and Lewis/

Lewis, 1968). The great efficiency of tagging of chicken erythrocytes with ^{51}Cr has been demonstrated by Jaffe and Hopkins (1961), Walter et al. (1965) and Wels et al. (1967), and its use in the present studies has revealed direct evidence of the increased erythrocyte destruction, and thus permitted an accurate expression of the rate of destruction relative to the normal value. It has also enabled an estimate to be made of the mean erythrocyte life-span associated patterns of in-vivo erythrocyte destruction during infection.

During experimental acute fowl typhoid the average haematocrit fell to about 50 per cent of its original volume (Chapter II). However, using the radiochromium labelling method to estimate blood cell loss during this period, the indications were that nearly 80 per cent of the infected animals' original total number of erythrocytes has been destroyed at the time of death (Table 19). These observations demonstrate that much greater numbers of erythrocytes are destroyed during the course of this infection than has hitherto been shown by standard haematological methods. This underestimate of the actual total erythrocyte numbers destroyed is clearly the consequence of compensatory erythropoiesis, which is replacing the lost erythrocytes with new, unlabelled, probably immature cells (reticulocytes). This is supported by histological studies which showed bone marrow hyperplasia and marked reticulocytosis (Chapters II and III). The use of the radiochromium method has therefore enabled the quantity of the original total of circulating erythrocyte number destroyed to be estimated.

The survival curves are regarded here as reflecting the actual/

actual life-span of the erythrocytes during infection. The total ^{51}Cr activity loss could not have been significantly influenced by the daily sampling, as each sample was only slightly more than 1 ml. and the total blood loss due to sampling was about 10 mls., corresponding to about 5 per cent of the total blood volume. Moreover, the finding that the rate of ^{51}Cr elution from labelled cells is neither altered in haematologic diseases (Read et al., 1954) nor significantly important in haemolytic processes where there is rapid destruction of erythrocytes (Necheles et al., 1953; Hughes-Jones and Mollison, 1956; Dacie and Lewis, 1968) lend further support to the conclusion that the survival curves are an accurate reflection of erythrocyte life-span during acute fowl typhoid.

Erythroclasis during this infection is very rapid and predominantly extra-vascular. This is indicated by the absence of any increase in plasma haemoglobin and measurements of radioactivity of most of the infected animals had also not shown any significant deviation from the background activity. Moreover, in the few infected chickens which had shown slight increases in plasma radioactivity, the levels rose very slowly, suggesting that ^{51}Cr is liberated into the plasma only after the labelled erythrocytes have been trapped in the RES. In addition, organ radioactivity measurements (Table 21) after post-mortem, confirm the earlier conclusion that the organs of the RES are the main depositories and eventual destroyers of the injured and sensitized erythrocytes during this infection (Chapters II and III). For instance, Table 21 shows that over 70 per cent of the total injected radioactivity was estimated to be localised in the spleen and liver alone. Each of these organs seems to have destroyed proportionately equal amounts/

amounts of labelled cells, although the spleen, if its total radioactivity is related to the activity per unit wet weight, may be the more important organ.

It has also been suggested earlier that the anaemia of fowl typhoid may be initiated primarily by the sensitization of the erythrocytes rather than by any possible stimulation of the RES (Chapters II and III). However, the behaviour of normal, homologous erythrocytes in infected chickens seems to contradict this assumption, in that normal cells are rapidly destroyed in these animals (Fig.34). Nevertheless, the strong possibility exists that these cells do in fact become sensitized in-vivo after being transferred to their new host. This remains undetermined, but this possibility is substantiated by the finding that chickens transfused with normal erythrocytes less than 24 hours before death could only destroy about a third of the labelled cells. In contrast, less than 10 per cent of the total injected labelled cells still remained when the animals were transfused with normal erythrocytes 2 or 3 days before death. This suggests that, in this instance, the cells have had sufficient time to be sensitized in-vivo and were therefore subsequently more rapidly eliminated. However, the possibility exists that the greater destruction of labelled erythrocytes may be a direct consequence of their prolonged association with, possibly, a hyperactive RES. As a corollary to these findings, in-vivo sensitized cells taken from infected chickens and injected into normal chickens, were also rapidly cleared (Fig. 35). This clearly indicated that RES hyperactivity cannot be an initiating factor. These results clearly show that it is the erythrocyte sensitization which is the primary initiating pathogenic factor/

factor in the haemolytic syndrome.

The finding that in-vivo Type III erythrocytes (that is, cells sensitized with immune globulins) were rapidly cleared was also contrary to what might have been expected from the reports of Loutit (1946), Mollison and Patterson (1949) and Lindsey, Donaldson and Woodruff (1966). These workers produced evidence to show that the presence of incomplete antibody on the surface of erythrocyte was not a sufficient condition for shortened cell survival and indeed, it may have a protective function. (Thomas, 1964).

The pathogenic mechanism suggested above for the induction of anaemia of this infection is supported by subsequent in-vitro experiments in which injection of erythrocytes, maximally sensitized with S.gallinarum LPS, into normal chickens also resulted in their rapid destruction (Fig.36), indicating clearly that, at least the presence of bacterial polysaccharide on the erythrocyte surface is a sufficient condition for its rapid destruction. The observations by Ceppellini and De Gregorio (1953) and Shumway, Bokkenheuser, Pollock and Neter (1963) of decreased survivals in rabbits injected with Vi and typhoid-O antigen-sensitized erythrocytes, support this conclusion. Furthermore, it was found that even though transfused, autologous, minimally sensitized cells were eliminated at a significantly lower rate than maximally sensitized cells, the rate of destruction was still significantly faster as compared with that of normal or sham sensitized erythrocytes. These observations are in agreement with the demonstration that erythrocytes whose surface has been physically or chemically altered in-vivo are rapidly phagocytosed by the RES when injected intravenously (Miescher, 1957; Jandl and Simmons, 1957; Mollison, 1962; Crome and Mollison 1964; Azen and Shilling, 1964; Ultman and Gordon, 1965; Kimber Lander/

Lander and Robson, 1965).

A further indication of the mechanism of the haemolytic anaemia of this infection, was obtained by the observation that when maximally sensitized erythrocytes were auto-transfused into primed (specifically immune) chickens, they were nearly all eliminated within minutes (Fig.37), and the surviving radioactivity was found to be mainly present in the plasma. This finding, coupled with the demonstration that autologous erythrocytes carrying the specific bacterial polysaccharide antigen are very rapidly eliminated from the circulation on the injection of homologous bacterial antibody (Figs.39 and 40 and Table 20), strongly indicated that the haemolytic episode associated with acute fowl typhoid is mediated by a specific immune mechanism. The results clearly establish that the fixation of salmonella polysaccharide by the erythrocytes during infection changes their surface in such a way as to greatly increase their rate of sequestration; and the subsequent active production of specific bacterial antibody enhances still further the rate of elimination of these in-vivo sensitized cells from the circulation. The eventual fate of the sequestered erythrocytes is their destruction by the RES — the cumulative outcome of all adverse physico-chemical changes in the erythrocyte surface (Rifkind, 1966; Leddy, 1966) — and in this instance, initiated by surface immunological reactions.

Therefore the present findings, in contrast to earlier observations of Biozzi and Stiffel (1962), and Biozzi (1968 - Personal communication), conclusively support the hypothesis that opsonin-promoted erythrophagocytosis plays a most significant part/

part in the rapid destruction of erythrocytes sensitized with bacterial antigens in-vivo, and hence, the haemolytic anaemia of this infection. This mechanism may also apply to haemolytic episodes observed during other gram-negative bacterial infections. Indeed, the above hypothesis is in agreement with the observation that immune, natural hetero- and iso-antibodies greatly increase the rate of destruction of both heterologous and homologous erythrocytes intravenously injected (Halpern, Biozzi, Benacerraf and Stiffel, 1957; Benacerraf et al., 1957; Hughes-Jones, Mollison and Veall, 1957; Jandl and Tomlinson, 1958; Cutbush and Mollison, 1958; Cutler, 1961; Mollison, 1961; 1962; Mollison and Hughes-Jones, 1967).

The results of radiochromium experiments discussed so far have shown that a reasonably accurate estimate of the intensity and nature of the haemolytic process can be made with some degree of certainty. Nevertheless, a more complete appreciation of the phenomenon of abnormal disappearance of isotope from the circulation of the treated animals, and the pattern of erythrocyte destruction, depends on the quantitative study of organ sites of isotope deposition. As Table 21 shows, the main conclusion to be drawn from the organ activity distribution studies is that the rate and pattern of erythrocyte destruction are directly dependent on the degree of sensitization; increased activity and physical architecture of the RES may only have been secondarily involved. Thus, hepatic sequestration generally involved the grossly agglutinated erythrocytes, where maximally sensitized erythrocytes were either inoculated into immune animals or were injected into normal/

normal animals and afterwards passively challenged with specific antibody. On the other hand, erythrocytes whose surfaces have been altered by either polysaccharide alone, or in association with non-agglutinating, incomplete antibodies, are preferentially filtered by the spleen. These potentially agglutinable cells (for example, maximally or minimally sensitized erythrocytes transfused into normal chickens) are eliminated relatively slowly as compared to antibody mediated clearance, and the spleen was invariably shown to be the main organ for destruction of these cells.

This characteristic pattern of organ radioactivity distribution and cell destruction may be explained by the reports of Jandl (1955), Hughes-Jones, Mollison and Veall (1957), Cutbush and Mollison (1958), Mollison and Hughes-Jones (1958) and Mollison (1962) who, using different experimental animal models, demonstrated that strongly agglutinated erythrocytes are rapidly removed mainly by the liver, whereas thinly-coated (incomplete antibody) non-agglutinated erythrocytes are sequestered comparatively slowly, and predominantly in the spleen.

Another important observation made in this study was the fact that survival curves of some of the clearance studies could be resolved into 2 components. For example, maximally sensitized erythrocytes, auto-transfused into normal or immune chickens, were found to be eliminated at two different rates — a fast and a slow component (Figs.36 and 37). There was, however, direct evidence, particularly from plasma radioactivity and haemoglobin measurements, that the longer surviving component was mainly due to ^{51}Cr elution from the erythrocytes. On the other/

other hand, the two component curves obtained when either Type II or III in-vivo sensitized cells from infected animals were transfused into normal chickens, appeared to indicate the presence of two populations of cells with different survival rates (Fig.35). This conclusion is supported by the findings that no plasma haemoglobin (haemolysis) was visible, and neither was any plasma radioactivity detectable at the crucial point where the 2 component-curves meet. Thus, the shorter surviving curve (with a mean cell-life of 4 days and maximum life-span of 6 days) may probably represent the clearance of in-vivo 'maximally' sensitized erythrocytes, whilst the longer surviving curve (with a maximum life-span of 28 days) may represent the clearance of non-sensitized erythrocytes (reticulocytes).

The suggestion that the two-component curves may be characteristic of haemolytic anaemias with intra-corpuseular defects (Dacie and Lewis, 1968) is supported by the finding that human patients with sickle-cell anaemia constantly exhibit this type of survival curve (Callender, Nickel, Moore and Powell, 1949; Singer and Fisher, 1952). However, the results of experiments in this study, where the clearance of small and large doses of in-vitro sensitized erythrocytes was determined (Fig. 39 and Table 20) indicate that one of the causes of the two-component curves may be 'exhaustion' of antibody as well as the uneven distribution of the sensitizing polysaccharide within the injected erythrocyte population. To obtain further evidence in support of this hypothesis, clearance studies involving two successive injections of specific antibody, after transfusion of a fairly large dose of labelled, maximally sensitized cells, were performed. The results clearly demonstrate that two/

two-component curves can be produced by exhaustion of specific antibody (Fig.40). They indicate, furthermore, that the degree of erythrocyte sensitization can influence the pattern of the survival curves.

Therefore, the finding that the elimination curves of some of these haemolytic episodes have two components has contributed to the elucidation of the mechanism of anaemia of fowl typhoid and also indicated that haemolysis is extra-vascular.

In preliminary experiments on in-vivo erythrocyte sensitization, reported later (Chapter VII), it was observed that a large dose of endotoxin, administered intravenously into normal chickens, induces erythrocyte sensitization in-vivo. It was also observed that sensitization could still be demonstrated up to 4 or 5 days after endotoxin injection, then a rapid elimination was observed to occur. This final cell destruction was such that a severe anaemia developed. The nature of the in-vivo sensitization and the timing of the appearance of specific haemagglutinating antibodies suggested that this episode of rapid clearance of cells may be due to antibody activity induced by the injected endotoxin. It was therefore decided to study this phenomenon, using radiochromium. The results, depicted in Fig.38, support the hypothesis that the anaemia which develops after endotoxin injection is haemolytic and that the mechanism may have an immunologic factor. This is supported by the observation that the anaemia developed at precisely the time when specific anti-polysaccharide antibodies appeared in the circulation. Prior to this critical period, in-vivo sensitization of erythrocytes was readily/

readily demonstrable, but soon after this period, sensitization was no longer detectable. The suggestion here is that the subsequent in-vivo combination of sensitized erythrocytes with the induced antibody accelerated their rate of elimination and destruction by the RES. This conclusion is supported by the findings that none of the animals developed observable intra-vascular haemolysis, and animals injected with the same large dose of endotoxin 6 or 24 hours before injection of labelled cells did not exhibit any significant shortening of erythrocyte curves (Fig. 38). These observations are at variance with the suggestion that non-specific increase in RES activity accounts for this type of haemolytic anaemia (Ho and Kass, 1958).

These are very interesting and striking observations, and the possibility that this type of immune haemolysis could serve as an ideal experimental model for the study of anaemia, especially of gram-negative bacterial infections, warranted further studies. The results of such experiments are embodied in Chapter VII.

In view of the earlier suggestion by Buxton (1959_a) that during acute fowl typhoid the erythrocytes are only partially sensitized in-vivo, the present finding that in-vivo Types II and III sensitized cells are destroyed very rapidly in normal chickens is surprising and very significant. This striking observation suggests that in-vivo sensitization caused more severe damage to the erythrocytes than has previously been imagined, and clearly indicates that the cells cannot be in a state of partial sensitization as suggested by Buxton (1959_a). The sensitized cells, recovered from infected animals, may consist of either a mixed cell population of 'maximally' or heavily sensitized erythrocytes and reticulocytes, or/

or a mixed population of cells showing varying degrees of sensitization, of which the maximally sensitized numbers have been eliminated, leaving only the partially sensitized cells to be detected. In support of these assumptions is this significant observation: when autologous erythrocytes, with minimal amounts of polysaccharide on their surface, were transfused into immune chickens, they were not rapidly cleared (Fig.37). Erythrocyte survival was only moderately altered as compared with sham-sensitized cells, and there was even not much difference in the rates of clearance of such cells in either immune or non-immune animals (Figs.36 and 37). However, the possibility exists that a more rapid elution of polysaccharide in the circulation may have occurred in this system. Nevertheless, observations of clearance rates of such cells in immune animals and in normal animals later challenged with homologous antibody (Fig.39C), make this latter possibility highly unlikely.

Evidently, if in-vivo sensitization was partial as has been suggested (Buxton, 1959 a) then it would be hard to understand how rapid antibody-mediated destruction of in-vivo altered erythrocytes could account for the anaemia of this infection, as has been postulated in the present studies — unless RES hyperactivity plays a more direct and active role than the present results seem to indicate.

The thesis that the cell population of in-vivo sensitized erythrocytes taken from infected animals may be heterogeneous with respect to the sensitizing factors — due probably to uneven quantitative distribution of specific polysaccharide — was experimentally tested in the following Chapter (VI).

CHAPTER VIIN-VIVO ERYTHROCYTE SENSITIZATION : EVIDENCE
FOR HETEROGENEITY OF THE ERYTHROCYTE POPULATIONINTRODUCTION

Investigations in previous chapters have shown that a severe anaemia is an important feature of fowl typhoid infection and that this coincides with a serologically detectable alteration of the erythrocyte surface. It has therefore been proposed that this sensitization leads to the immune elimination of the erythrocytes, thus giving rise to the anaemia. Subsequent radioisotopic studies indicated that an essential prerequisite of rapid immune clearance was that it was necessary for the cells to be heavily (that is, maximally) sensitized, as it was found that partially sensitized cells were only eliminated slightly more rapidly than normal, non-sensitized erythrocytes. It was, however, difficult to reconcile this observation with the finding that in-vivo sensitized cells were only detectable by an antiglobulin haemagglutination procedure which therefore suggested that these cells were only partially or minimally sensitized.

One possible explanation of this anomaly was that two populations of cells existed :

- (1) heavily sensitized cells present in low numbers as a consequence of rapid clearance, and
- (2) a larger population of normal, unsensitized erythrocytes (probably reticulocytes). Such a heterogeneous cell population/

population would thus account for the weak haemagglutinating activity. This hypothesis is, however, at variance with the suggestion of Buxton (1959a) that such cells are partially and uniformly sensitized.

Another possibility was that varying degrees of erythrocyte sensitization occurred and that all maximally sensitized erythrocytes were rapidly removed, leaving only the partially sensitized cells in the circulation. This would account for the weakly positive ICT. According to this hypothesis then, all the recovered cell population would be sensitized, whereas in the first only a proportion would be in this state.

The studies presented in this chapter were therefore designed to investigate the validity of the hypotheses offered above.

MATERIALS AND METHODS

Hyperimmune serum: Hyperimmune anti-S.gallinarum -O serum was produced in adult chickens by multiple injections of heat-killed organisms (6 minutes at 100°C).

Indirect Antiglobulin Haemagglutination test (ICT) and

Haemagglutination (HA) Test: The techniques of these tests were as described previously (Chapters II and III). Chicken erythrocytes were sensitized with alkali-treated lipo-polysaccharide (LPS), and the methods of alkali-treatment and erythrocyte sensitization have also been described earlier.

Macroscopic Examination of Haemagglutination Patterns: The haemagglutinating patterns of mixed population of erythrocytes, consisting/

consisting of various proportions of in-vitro maximally sensitized and non-sensitized (normal) cells, and obtained with the ICT and HA test, were examined by gross visual inspection.

Approximately 1 ml. of washed, packed normal chicken erythrocytes were maximally sensitized in-vitro with alkali-treated LPS. After washing, a 2 per cent suspension in saline was made up; the same percentage saline suspension of normal erythrocytes was also made up. Then the two cell suspensions were mixed in various proportions, and equal amounts (0.2 ml.) of each mixed cell suspension were titrated against serially diluted homologous hyperimmune serum in an ICT. The anti-chicken γ -globulin serum was used at a standard dilution of 1:30.

Microscopic Examination of Haemagglutination Patterns: Preliminary experiments showed that a mixed population of erythrocytes, consisting of between 92 and 94 per cent of normal cells and 6 and 8 per cent of maximally sensitized erythrocytes, gave the same range of positive ICT titres as usually obtained with in-vivo sensitized erythrocytes taken from chickens infected with acute fowl typhoid.

These cells were taken and then subjected to haemagglutination (HA) test. All were negative, and the HA 'buttons' in the first few tubes were gently washed, once, in saline. After re-suspension in saline, a wet preparation of a small amount of this was mounted for microscopic examination and for photography.

Similar wet slide films of the following cell preparations, after reacting with the hyperimmune serum, were also/

also made:

- (i) In-vitro minimally sensitized erythrocytes.
- (ii) In-vitro maximally sensitized erythrocytes.
- (iii) Control, non-sensitized (normal) erythrocytes.
- (iv) In-vivo Type II sensitized erythrocytes, recovered from S.gallinarum infected chickens.

Electrophoretic Examination of Mixed Population of Erythrocytes:

The technique for the determination of electrophoretic mobility of erythrocytes has been fully described earlier (Chapter IV).

Various preliminary studies were conducted with different concentrations of sucrose, pH, sodium-potassium buffer and percentages of erythrocyte suspension, with the object of establishing ideal conditions for electrophoretic separation of sensitized and non-sensitized cells. Such separation will enable visual examination and subsequent measurement of the mobility of the different cell population to be made.

The results of such experiments showed that the best conditions for the electrophoretic separation of a mixed (50:50) erythrocyte population was in a 20 per cent sucrose-buffer, with a pH of 7.4.

Electrophoretic mobility analyses were subsequently carried out using these cell preparations:

- (i) Equal amounts (50:50) of maximally sensitized and normal erythrocytes were mixed together and suspension of 2.5; 5; and 10 per cent in 20 per cent sucrose-buffer were then electrophoresed.
- (ii) Equal amounts of minimally sensitized and normal cells were also mixed and electrophoresed under the same conditions as/

as in (i).

(iii) 5 per cent suspension of normal and maximally sensitized erythrocytes, which had been combined in a proportion of 92 and 8 per cent respectively, — the adjusted mixed cell ratio which gives approximately the same ICT titre as obtained with in-vivo sensitized erythrocytes taken from infected chickens — was electrophoresed suspended in 20 per cent sucrose-buffer.

(iv) 5 per cent suspensions in 20 per cent sucrose-buffer of in-vivo Types II and III sensitized erythrocytes were also electrophoresed.

The sucrose-buffer was used both to wash and suspend the cell preparations for electrophoresis.

The mean values of both the electrophoretic velocity and mobility for each preparation were determined.

Terminology: Preliminary experiments indicated that once a critical proportion of the erythrocyte LPS-receptor sites had been occupied by LPS molecules, and depending on the potency of the homologous antiserum, haemagglutination readily occurs when sensitized cells and the antibody are reacted together. Similar observations have been made by Lüderitz, Westphal, Sievers, Kröger, Neter and Braun (1958) and Bokkenheuser and Koornhof (1959). For instance, the former authors demonstrated that even though approximately 100,000 molecules of E.coli alkali-treated LPS were needed to maximally sensitize each single erythrocyte, fixation of only 5,000 molecules suffices for positive haemagglutination (HA).

Therefore, when in the following studies it is suggested/

suggested that a portion of the in-vivo sensitized cells taken from infected animals is 'maximally' sensitized, it will not imply the total occupation of all the LPS - receptor sites by the sensitizing LPS molecules. The term is used here simply to denote erythrocyte adsorption of a sufficient quantity of LPS which would suffice for positive direct HA.

RESULTS

(1) Relative Sensitivity of ICT and HA Test in the Detection of Mixed Populations of Erythrocytes:

The inability of the HA test, visually, to detect the presence of maximally sensitized cells below a certain level in a mixed population with normal, non-sensitized erythrocytes is shown in Fig.41. Clearly, when the ratio of sensitized to normal cells is decreased below 1 to 9 respectively, the HA test is incapable, macroscopically, of demonstrating the presence of the sensitized cells.

Maximally sensitized and normal erythrocytes in a ratio of 8 to 92 respectively had been found to give approximately the same ICT titre as in-vivo sensitized cells taken from infected animals. When this mixed population of cells was subjected to the HA test and examined visually, no agglutination could be detected. However, the ICT gave positive reactions to mixed cell populations consisting of maximally and non-sensitized cells in a proportion as low as 1 in 39 respectively (Fig.41). Nevertheless, even though the ICT can visually demonstrate general sensitization to that extent, it cannot distinguish/

SENSITIVITY OF THE I.C.T. IN THE DETECTION OF MAXIMALLY SENSITIZED ERYTHROCYTES, MIXED

WITH NORMAL CELLS																			
Percentage Proportions Contained in Mixed Population of 2% Suspensions of Chicken Erythrocytes		DILUTION		OF		SALM.		GALLINARUM		ANTISERUM									
Normals R.B.C.	Maximally Sens. R.B.C.	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	1 2048	1 4096	1 8192	1 16384	1 32768	1 65536	1 131072	
0	100																		
50	50																		
90	10																		
95	5																		
96.5	3.5																		
97.5	2.5																		
100	0																		
<div><div></div><div></div><div></div></div>																			
POSITIVE HAEMAGGLUTINATION																			
" " ANTIGLOBLIN HAEMAGGLUTINATION																			
NEGATIVE " "																			

FIGURE 41. Relative Sensitivity of ICT and HA Test in the Detection of Mixed Populations of Erythrocytes.

distinguish or differentiate between maximal and minimal degrees of sensitization by simple macroscopic examination. The ICT even has a restricted sensitivity visually, since no agglutination was observed when the proportion of maximally sensitized to normal cells was less than 1 to 49 respectively.

(2) Microscopic Evidence for Heterogeneity amongst Erythrocyte Population:

(i) Evidence from in-vitro Sensitized Erythrocytes: After the failure, visually, by the HA test to detect the 8 per cent maximally sensitized cells in a mixed population with 92 per cent normal cells (that is, the cell mixtures adjusted to give ICT titre comparable to in-vivo sensitized cells), the same mixture was examined microscopically after being reacted with homologous antiserum. The photomicrograph of a wet preparation of such cells is shown in Fig.42. As suspected, clumps of agglutinated erythrocytes were seen scattered throughout the wet preparation. The agglutinates were rather small, and not as big as those examined after maximally sensitized cells were incubated with homologous antiserum (Fig.43). There are clear distinguishing differences between these two preparations. For example, where only maximally sensitized cells were examined after agglutination, only a few individual cells were left unagglutinated; the whole preparation consisted of large clumps of agglutinates. In contrast, with the mixed-cell preparation, substantial numbers of small agglutinates, dispersed amongst numerous unagglutinated single cells, could be clearly distinguished.

In direct contrast to the above observations, cells which/

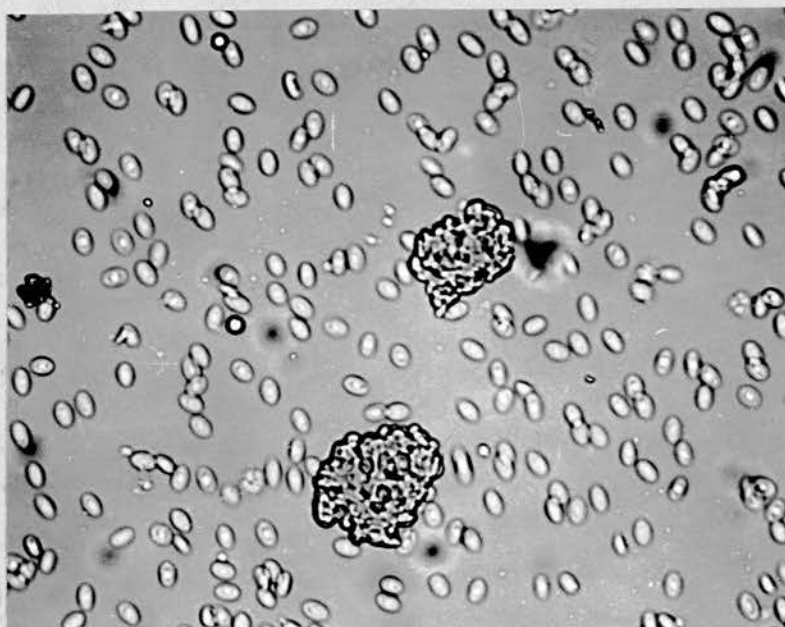


FIGURE 42. Photomicrograph of a wet film of a mixture of normal and in-vitro maximally sensitized erythrocytes in a ratio of 92:8 resp., after reaction with homologous antiserum. Several clumps of small agglutinates of erythrocytes dispersed amongst numerous unagglutinated, single cells. X 185 approx.

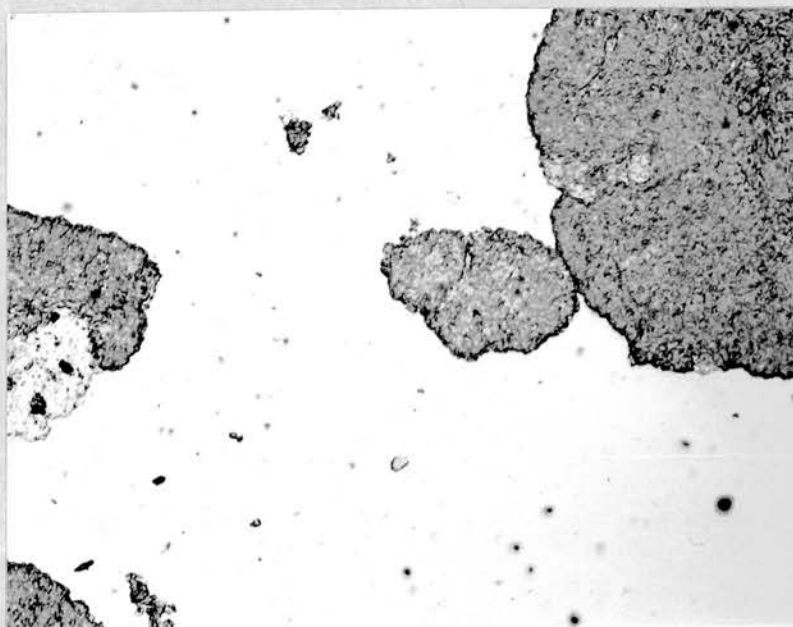


FIGURE 43. Photomicrograph of a wet film of in-vitro maximally sensitized erythrocytes after being subjected to HA test. There are large clumps of haemagglutinates and few individual erythrocytes. X65 approx.

which had been either sham-sensitized or sensitized minimally with LPS, did not exhibit any agglutination after being incubated with homologous antibody (Figs.45 and 44 respectively). However, the suspension of minimally sensitized cells showed a slightly greater clumping than the sham-sensitized cells (Fig.44), suggesting that, in fact, there was some degree of weak agglutination occurring. This appeared to affect all cells to the same extent.

(ii) Evidence from in-vivo Sensitized Erythrocytes: Erythrocytes sensitized in-vivo were obtained from infected chickens after detection with ICT in the standard manner. When such cells were incubated in homologous antiserum (HA test) agglutination could be demonstrated microscopically (Fig.46). On gross visual inspection after reacting with S.gallinarum antiserum, these erythrocytes did not appear to be agglutinated by the antiserum, but microscopic examination showed sizeable clumps of agglutinates scattered throughout the preparation. The agglutinates were, however, relatively smaller; apparently not so many erythrocytes were involved in positive haemagglutination as those agglutinates described above for cell populations involving larger amounts of in-vitro maximally sensitized cells.

However, the striking similarities of pattern of agglutination and sizes of agglutinates observed between cell mixtures adjusted to give an ICT titre comparable with in-vivo sensitized cells (8:92) strongly suggest that the in-vivo sensitized erythrocytes taken from infected chickens are heterogeneous.

(3) Evidence based on Electrophoretic Mobility Studies:

The overall summary of results is shown in Table 22.

(i) /

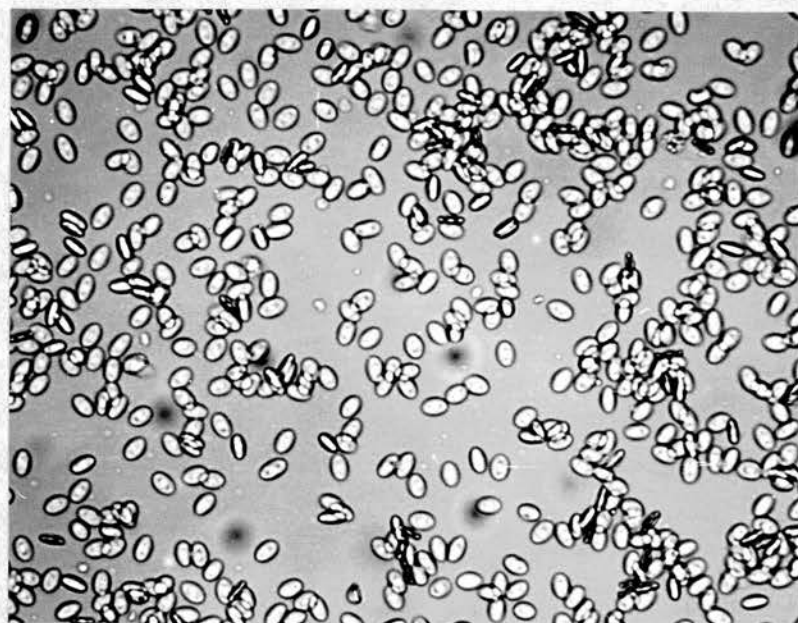


FIGURE 44. Photomicrograph of a wet film of in-vitro minimally sensitized erythrocytes after incubation with homologous antibody. No obvious agglutinates present, but slight clumping may indicate some degree of weak agglutination, affecting all erythrocytes to the same extent. X185 approx.

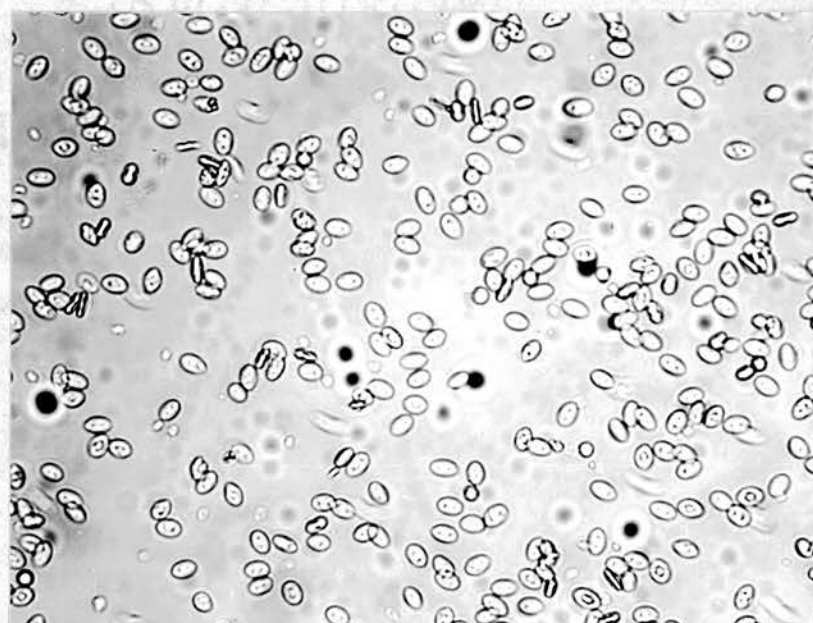


FIGURE 45. Photomicrograph of a wet film of sham-sensitized erythrocytes after reaction with homologous antibody. Absence of haemagglutination.

X185 approx.

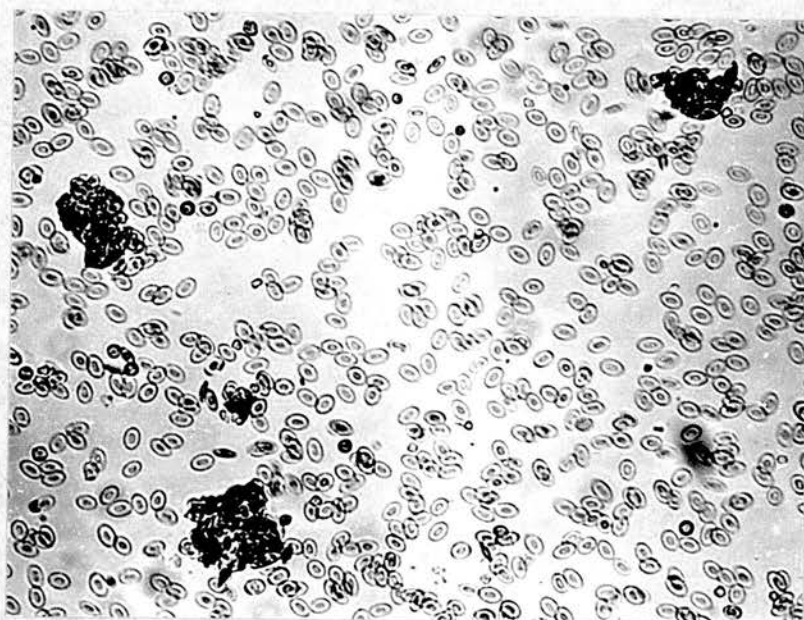


FIGURE 46. Photomicrograph of a wet film
of erythrocytes taken from
S.gallinarum infected chicken
after being incubated in
homologous antiserum. (The
cells had previously been shown
to be Type II sensitized
erythrocytes). Heterogeneity is
demonstrated by the presence of
sizeable clumps of agglutinates
scattered throughout the
preparation.

X140 approx.

(i) Electrophoretic Separation of Mixed-Population of in-vitro Sensitized and Non-sensitized Erythrocytes: Results of preliminary studies showed that maximally sensitized and non-sensitized erythrocytes, mixed in various proportions, could be electrophoretically separated into two distinct columns of differing mobility which enables their respective mobility values to be determined with comparative ease.

It was found that electrophoresis of a 2.5 per cent suspension of equal amounts of normal and maximally sensitized erythrocytes (50:50) in 20 per cent sucrose-buffer did not result in obvious visible separation of the different cell populations. However, when either 5 or 10 per cent mixed suspension of these two different cell preparations was electrophoresed under the same conditions as above, a clear demarcation between the cell populations was observed : a slow and a fast moving cell column. The mean electrophoretic velocity for the slow-moving column was estimated to be 16.6 mm. per hour, with a corresponding mean electrophoretic mobility of $0.49\mu/\text{sec.}/\text{volt}/\text{cm}$. The corresponding electrophoretic velocity and mobility values for the fast-moving cell column were also determined to be 31.6 mm. per hour and $1.03\mu/\text{sec.}/\text{volt}/\text{cm}$. respectively.

Comparison of the above values with the electrophoretic values obtained for maximally sensitized and normal erythrocytes (Table 22) lead to the conclusion that the slow-moving column consisted mainly of maximally sensitized cells, whereas the fast-moving column was made up of non-sensitized (normal) erythrocytes.

Of more significance was the finding that when cell mixtures/

TABLE 22

ELECTROPHORETIC MOBILITY DETERMINATIONS AS 5% CELL SUSPENSIONS IN 20% SUCROSE-BUFFER

ERYTHROCYTE PREPARATION FOR ELECTROPHORESIS	PERCENTAGE PROPORTIONS CONTAINED IN MIXED ERYTHROCYTE SUSPENSION		TOTAL NUMBER OF DETERMINATIONS	MEAN ELECTROPHORETIC VELOCITY (mm/hour)	MEAN ELECTROPHORETIC MOBILITY ⁺ (μ /sec/volt/cm)
	NON-SENSITIZED (NORMAL)	MAXIMALLY SENSITIZED			
MIXTURE OF NON-SENSITIZED (NORMAL) AND MAXIMALLY SENSITIZED	92	8	5	15.8	$0.47 \pm 0.031^*$
				31.8	$1.02 \pm 0.009^{**}$
IN-VIVO SENSITIZED CELLS FROM CHICKENS INFECTED WITH ACUTE FOWL TYPHOID	TYPE II SENSITIZED		6	17.2	$0.52 \pm 0.028^*$
				30.3	$1.00 \pm 0.042^{**}$
				16.4	$0.49 \pm 0.016^*$
				31.0	$1.01 \pm 0.040^{**}$
NON-SENSITIZED (NORMAL)	100	0	3	32.0	1.03
MAXIMALLY SENSITIZED	0	100	3	16.0	0.48
MINIMALLY SENSITIZED		100	2	30.0	0.96

⁺ = MOBILITY VALUES EXPRESSED AS MEAN \pm S.E., WHERE APPROPRIATE.^{*} = SLOW-MOVING ERYTHROCYTE COLUMN^{**} = FAST-MOVING ERYTHROCYTE COLUMN

mixtures, consisting of maximally sensitized and normal erythrocytes and adjusted to give ICT titre comparable to in-vivo sensitized cells (8:92), were electrophoresed, two distinct migrating cell columns were observed. The mobility of the slow-moving cell column, which probably consisted mainly of maximally sensitized cells, had approximately the same value ($0.47\mu/\text{sec.}/\text{volt}/\text{cm.}$) as the value obtained for maximally sensitized cells alone ($0.48\mu/\text{sec.}/\text{volt}/\text{cm.}$) (Table 22). The fast-moving column had, significantly, the same mobility ($1.02\mu/\text{sec.}/\text{volt}/\text{cm.}$) as the value obtained for normal cells ($1.03\mu/\text{sec.}/\text{volt}/\text{cm.}$).

In direct contrast, when minimally sensitized and normal cells were mixed in equal proportions (50:50) and the same range of suspensions in sucrose-buffer as used for maximally sensitized cells above, subsequent electrophoresis did not result in the separation of the different cell preparations. The mean electrophoretic mobility of this mixed cell population ($1.01\mu/\text{sec.}/\text{volt}/\text{cm.}$) was only slightly different from the mean value obtained for normal erythrocytes ($1.03\mu/\text{sec.}/\text{volt}/\text{cm.}$) (Table 22).

(ii) Electrophoresis of Sensitized Erythrocytes obtained from Infected Chickens: It was found that erythrocytes obtained from infected chickens, and giving positive ICT on electrophoresis, again separated with two distinct columns which appeared identical to the result achieved using a mixed cell population of maximally sensitized and normal erythrocytes (8:92). The mobilities of the slow-moving cell columns were very significantly decreased as compared to the normal cell values (Table 22). The fast moving column was also observed to have a slightly lower mobility than normal/

normal cells, but this discrepancy was considered insignificant if it is noted that generally, lower values were obtained throughout the present studies than in previous studies (Chapter IV). The lower electrophoretic values may be due to the doubling of the concentration of the sucrose solution, and hence, the proportional increase in the ionic strength of the sucrose-buffer solution.

An additional electrophoretic finding was the significantly lower mobility values obtained for the slow-moving cell column when in-vivo Type III sensitized cells were electrophoresed: $0.49 \pm 0.016 \mu/\text{sec.}/\text{volt}/\text{cm.}$, as compared with a corresponding value of $0.52 \pm 0.028 \mu/\text{sec.}/\text{volt}/\text{cm.}$ for in-vivo Type II sensitized cells (Table 22).

The results of these electrophoretic mobility experiments offer strong, direct evidence in favour of the suggestion of heterogeneity of the in-vivo sensitized cells. The electrophoretic separation of these cells into two columns with different mobility values has enabled their probable identities to be established.

DISCUSSION

Serologic, microscopic and electrophoretic evidence presented here are clearly in agreement with the hypothesis that erythrocytes, recovered from chickens injected with acute fowl typhoid, may in fact be heterogeneous with respect to the quantity of the sensitizing bacterial polysaccharide. These cells may be a mixture of non-sensitized cells (probably reticulocytes) and a minority of cells which are either 'maximally' or heavily sensitized to/

to give a positive direct haemagglutination with the homologous antibody.

The present observations are clearly at variance with the earlier conclusions of Buxton (1959a). This author had inferred that in-vivo sensitization had only occurred to a partial extent during fowl typhoid because sensitization of erythrocytes could only be detected by an antiglobulin haemagglutination test (ICT) and not by a direct haemagglutination (HA) test. The implications of this conclusion, which have also been made by Young and colleagues (Young et al., 1962) in their studies on infantile diarrhoea, are that in-vivo erythrocyte sensitization involved minimal or small quantities of adsorbed polysaccharide, and that sensitization was uniformly spread throughout the recovered erythrocyte population. The evidence obtained from the present study clearly does not support these assumptions. The inability of the ICT to differentiate between maximal and minimal (partial) degrees of sensitization is clearly shown by Fig.41.

Macroscopically, the direct HA test is very insensitive, and cannot detect the presence of maximally sensitized cells below a certain threshold in a mixed population with non-sensitized cells (Fig.41). However, the ICT is relatively more sensitive than the HA test, but even this test has been found to have a restricted sensitivity. Thus, Ceppellini and De Gregorio (1953), using the ICT, observed that erythrocytes, maximally sensitized with Vi-antigen, survived for only 28 hours in non-immune rabbits. And when Buxton (1960) withdrew nearly a third of the total blood volume each from a group of normal chickens, and maximally sensitized the erythrocytes with S.gallinarum LPS, and then re-transfused them/

them into the original animals, he could detect no trace of these sensitized cells 30 minutes after re-inoculation by using the following tests: the ICT; a double-antiglobulin haemagglutination test; and a mixed antiglobulin haemagglutination test, similar to the one described by Jones and Silver (1958). However, results of radiochromium-labelling studies (Chapter V) have demonstrated clearly that maximally sensitized cells could still be detected 4 to 6 days after auto-transfusion into normal animals. And recently, Shumway (1964) had found that detection of maximally sensitized erythrocytes, with typhoid O-antigen could be achieved with Mollisons' direct differential agglutination method (Mollison, 1967) only when the proportion of sensitized to normal erythrocytes was not less than 1 to 20 respectively.

The suggestion that in-vivo erythrocyte sensitization is "maximal", and that some of these cells would be recovered with the reticulocytes at the critical time of sampling for sensitization determination is again amply supported by the following observations: When the washed erythrocytes from the infected animal were reacted with S.gallinarum antiserum no agglutination was discernible on gross visual examination. However, examined microscopically, sizeable agglutinates could be seen scattered throughout the wet preparation (Fig.46). Similar observations were recorded when in-vitro maximally and normal cells — mixed in a ratio (8:92 respectively) to give the same ICT titre as normally determined for in-vivo sensitized cells — were also examined microscopically (Fig.42). Thus, visually the HA test could not detect the presence of these maximally sensitized cells; microscopically, however, it demonstrates that/

that the cells are more than partially sensitized, and may even be maximally sensitized in-vivo. The ICT can visually demonstrate the presence of in-vivo sensitization, but its previous use has not been of value in demonstrating the extent and the quantitative degree to which the cells are sensitized in-vivo.

Previous indirect evidence indicating that in-vivo sensitization may involve erythrocyte adsorption of much greater quantities of bacterial LPS — sufficient to give a positive HA — than has hitherto been considered to occur, was the highly significant observation made during the immunofluorescent studies recorded in Chapter IV. Under the fluorescent microscope, the minimally sensitized cells were seen as a collection of numerous individual erythrocytes, with a few or no fluorescent agglutinates present (Fig.28). This uniform, homogeneous fluorescence of minimally sensitized cells was in direct contrast to the slightly heterogeneous fluorescent agglutinates observed when wet preparations of in-vivo sensitized cells were examined. These cells exhibited a combination of few individual cells with sharp, clear outlines, and numerous, but relatively small, fluorescent agglutinates, also with well-defined outlines (Fig.31). These observations probably indicated that the in-vivo sensitized cells had adsorbed much greater amounts of specific polysaccharide than previously believed — certainly much greater than minimal amounts.

Again, in Chapter IV, it was shown that the presence of LPS on the surface of the erythrocyte greatly reduced its electrophoretic mobility. The results of the present electrophoretic studies have also demonstrated that the erythrocyte population extracted from the infected animal at the peak of haemolytic crisis, and also serologically shown to be sensitized in-vivo, is electrophoretically/

electrophoretically heterogeneous (Table 22). Two distinct migrating cell columns of different mobilities, could easily be discerned. Estimation of the electrophoretic mobilities of these two columns clearly indicates that the slow and fast moving cell columns consist, respectively, of "maximally" and non-sensitized erythrocytes. This conclusion is supported by the finding of identical electrophoretic mobility values obtained for in-vitro maximally sensitized and normal erythrocytes, which had been previously mixed in a ratio of 8 to 92 respectively. In direct contrast, when minimally sensitized and normal cells were mixed, even in equal proportions, and then electrophoresed, no separation of the different cell preparations could be achieved. The mean electrophoretic mobility was not even significantly different from the value for the normal cell (Table 22), and the mobility value for wholly minimally sensitized cells was still below the combined value of in-vivo sensitized cells (Chapter IV). These results offer another convincing evidence in support of the contention that during acute fowl typhoid the erythrocytes adsorb sufficient quantities of bacterial polysaccharide to be directly agglutinated by homologous antibodies.

The results of the experiments recorded herein clearly demonstrate that, contrary to the earlier assumption of homogeneity and the partial extent of in-vivo sensitization, these cells are in fact heterogeneous with respect to the coating lipo-polysaccharide. It is suggested that a proportion of the extracted or recovered cells may be adequately or "maximally" sensitized. It is concluded that the latter cells are the remaining sensitized cells which persist in the circulation after the greater part of them had been eliminated, and rapidly destroyed during the short period of acute haemolytic crisis; /

crisis: a process greatly enhanced by the appearance of opsonising antibodies. It is also suggested that as a result of the inevitable mixing in the circulation of these two different cell populations, subsequent visual, or macroscopic, detection of the in-vivo erythrocyte sensitization is possible only by the use of the indirect antiglobulin haemagglutination test (ICT). Microscopically, in-vivo sensitization can be demonstrated with the direct HA test, indicating the probable "maximum" extent to which in-vivo sensitization occurs during acute fowl typhoid. In any case, for the erythrocytes to be rapidly destroyed, and therefore to account for the anaemia associated with this infection, they probably have to be "maximally" sensitized. Indeed Shumway, Bokkenheuser, Pollock and Neter (1963) have shown that the rate of clearance of sensitized erythrocytes is dependent upon the quantity of the adsorbed polysaccharide. The above postulated mechanism for this anaemia is supported by the finding that in both non-immune and immune chickens the presence of minimal amounts of polysaccharide on erythrocyte surface does not appreciably alter the erythrocyte survival rate, even at a time when homologous antibodies, engendered by these sensitized cells, had made their appearance in the circulation (Chapter V). Indeed, in the previous chapter, heterogeneity of the in-vivo sensitized cells had been demonstrated by the finding that 70 per cent of the ⁵¹Cr labelled - in-vivo sensitized cells were rapidly destroyed in a normal animal with a maximum cell life of 6 days; the remaining 30 per cent survived normally, with a maximum life-span of 28 days (Fig.35).

CHAPTER VIIEXPERIMENTAL INDUCTION OF A HAEMOLYTIC ANAEMIA IN THE
CHICKEN WITH S.GALLINARUM LPS.INTRODUCTION

Alterations in the numbers of circulating white cells and platelets after injections of gram-negative bacterial endotoxins (LPS) are well known (Thomas, 1954; Atkins, 1960; Braun and Landy, 1964; Zweifach and Janoff, 1966; Nowotny, 1969). Corresponding changes in the red cells, however, have not been clearly defined, and seems to vary from species to species. Thus, the chicken shows a decrease in haematocrit (Jordan and Hinshaw, 1964) instead of the commonly observed haemo-concentration in the dog (Gilbert, 1960; Kuida, Gilbert, Hinshaw, Brunson and Visscher, 1961) and bovine (Penhale, 1965; Tikoff, Kuida and Chiga, 1966). The monkey also shows a decrease in haematocrit (Hinshaw, Jordan and Vick, 1961) but Braude, Carey and Zalesky (1955) had found variable effects of E.coli LPS on the blood volume and haematocrit of rabbits, with little change in the circulating red cell volume, although leucopaenia occurred.

Previous preliminary experiments (Chapter V) have shown that in-vivo sensitization of erythrocytes with LPS is possible in chickens, but a relatively large dose of LPS has to be injected intravenously. Small amounts of LPS injected intravenously into animals have little or no effect on erythrocytes, and in fact, may be rapidly eliminated from the circulation (Braude et al., 1955; Rowley, Howard and Jenkin, 1956; Carey, Braude and Zalesky, 1958; /

Zalesky, 1958; Wiznitzer, Better, Rachlin, Atkins, Frank and Fine, 1960; Herring, Herrion, Walker and Palmer, 1963). For instance, 2 mgm. doses of LPS injected intravenously into 2 week old chickens hardly elicited any visible reactions (Cole and Boyd, 1965). Indeed, the chicken has long been known to be outstandingly tolerant to the toxic effects of LPS and can withstand unusually large doses of this toxin without apparent ill-effects (Cameron and Rettger, 1934; Landy and Johnson, 1955; Smith and Thomas, 1956; Heilman and Bart, 1964; Jordan and Hinshaw, 1964; Berczi, Bertok and Bereznay, 1966; Truscott and Inniss, 1967).

It may well be that the phenomenon of in-vivo sensitization, detectable so readily in the chicken, is dependent upon this property of refractoriness which would permit the build up of sufficient free LPS in the circulation to sensitize the erythrocytes. In other species, on the other hand, the much greater sensitivity to LPS may result in death before sufficient quantities are available for sensitization to occur.

As far as the author is aware, conditions have not so far been defined, experimentally, in any species whereby the injection of LPS and homologous antibodies have led to a reproducible haemolytic syndrome. Therefore attempts have been made to induce a haemolytic anaemia in the chicken by the injection of graded doses of LPS, and in some cases LPS together with subsequent passively administered homologous antiserum, in an attempt to further elucidate the role of both LPS and immunological mechanisms in the production of the anaemias of the natural infection.

MATERIALS AND METHODS

Animals: Over 80 ten-week old White-Link chickens, of mixed sex, were used in these experiments. They were housed, fed and maintained as described previously (Chapter II).

Preparation of LPS for injections: A large quantity of purified, lyophilised Westphal's lipo-polysaccharide (LPS) was prepared from S.gallinarum bacteria by the method described previously (Chapter III). It was accurately weighed and dissolved in a minimum of known volume of sterile phosphate-saline (pH 7.2), in order to obtain a standard of highly concentrated solution of LPS for all injections. The prepared LPS solutions were distributed into smaller quantities in Bijou bottles and stored at -20°C until required.

Production of Specific Hyperimmune serum: Specific anti-S.gallinarum LPS serum was produced in 3 ways and subsequently pooled:

(i) The procedure, previously described in Chapter V, was used, except that doses of the heat-killed bacteria were administered once, either intravenously or intramuscularly, to a number of adult White-Link chickens. The animals were bled at the end of 12 to 14 days after injection. A few of these immunized animals were re-immunized with varying doses of the killed bacteria, and sera from these animals were harvested by heart puncture, after being rested for 5 to 7 days.

(ii) 1 ml. of heat-killed bacterial suspension, containing 4.5×10^9 organisms per ml. and incorporated into complete Freund's adjuvant, was inoculated into the two sites of the chest muscles; it was boosted after one week with the same dosage, and bled 4 weeks later.

(iii) /

(iii) A group of chronically infected chickens of the same breed as used above — 3 to 4 months after surviving sub-acute fowl typhoid, and found to be clinically normal — were inoculated intramuscularly with different doses of the heat-killed S.gallinarum. Sera from these animals were harvested 6 to 9 days afterwards.

Sera from all the above groups of immunized chickens were pooled, the final haemagglutinating titre determined and then stored until required.

Collection of Blood samples: Blood samples were collected and assayed for alterations of the following parameters:

- (i) Haemoglobin concentration
- (ii) Packed cell volume (haematocrit)
- (iii) Reticulocyte levels
- (iv) In-vivo erythrocyte sensitization (Types II and III)
- (v) Specific antibody response — for both haemagglutinating (HA) and antiglobulin HA antibodies.

The methods for assay of the first 4 parameters above were the same as described previously (Chapter II). The fifth parameter was, however, determined by the procedures outlined in Chapter III.

The initial haematocrit values of individual chickens used for some of the experiments varied greatly, and in order to allow a direct comparison to be made between individuals, the percentage change of each sample (H_t) compared with the mean of pre-injection samples (H_o) was calculated as follows:

$$\text{Percentage haematocrit change} = \frac{H_t - H_o}{H_o} \times 100$$

For/

For the assay of specific O-antibodies in the samples collected, individual serum samples were coded by a second party and stored until later. At the end of all the experiments, the antibody levels in the stored, coded sera were determined 'blind'. Afterwards, the sera were decoded, and titres were plotted as Log_{10} reciprocals of serum dilutions.

Clinical examination : The clinical signs after injection of a single, large dose and also after challenge of LPS-injected chickens with homologous antiserum, were recorded. These included the external appearance and observations on temperature and respiratory responses.

Experimental design and Protocol : The immunological status of all the chickens to be used were determined before the start of the experiments. Selected animals, weighing between 800 and 900 gms. each, were then divided into 3 main groups.

Group I : Preliminary experiments conducted with groups of chickens had shown that a single standard dose of 40 mgm. per Kgm body weight of intravenously administered LPS was the maximum amount which regularly sensitized the erythrocytes in-vivo; in addition, this dosage did not produce any adverse clinical signs of endotoxin shock. (This dose of LPS will henceforth be referred to as the 'sensitizing dose' — S.D.).

About a dozen chickens were intravenously injected with S.D. of LPS, and blood samples taken daily were assayed for haematologic and serologic changes; the other parameters examined are enumerated above. An equal number of control chickens received the same volume of sterile saline instead of LPS.

Group II :/

Group II : The design of LPS injections in this group was such that it simulated the conditions in the disease syndrome in terms of periodic release of different amounts of LPS in-vivo. Therefore, this group of animals was divided into 3 sub-groups, and given various multiple sub-lethal doses of LPS as follows:

The first sub-group of animals received 10 mgm. of LPS per Kgm. daily for 7 days, and then on each alternate day for another 4 days.

The second and third sub-groups of animals received 20 mg. and 40 mg. per Kgm. of LPS respectively; The same schedule as above was followed. However, the latter sub-group of animals, initially, were given smaller doses on the first two days in order to gradually introduce them to the subsequent larger doses.

Each sub-group consisted of not less than a dozen chickens, of which approximately a third were used for control experiments. Control animals in this group received sterile saline instead of LPS.

Group III : In all, 24 'LPS-injected', and 5 'saline-injected' control, chickens were used for the demonstration of the effect of passive antibody on the clearance of experimentally induced in-vivo sensitized erythrocytes. 20 of the 'LPS-injected' animals were divided into 4 sub-groups, consisting of 5 chickens each.

On the detection of an adequate degree of erythrocyte sensitization (usually 2 hours after LPS injection) the following procedure was carried out:

The first sub-group of 5 chickens were challenged with homologous antiserum — 5 mls. per Kgm. of potent, undiluted hyperimmune /

hyperimmune serum (HA titre of 1:32,768); that is, 1 ml. antiserum for approximately every 200 gms. body weight of chicken. The second, third and fourth sub-groups of LPS-injected chickens were similarly injected with the same volume of specific hyperimmune serum, diluted 1:10, 1:50 and 1:100 respectively.

Of the remaining 10 chickens, half of these were similarly injected with S.D. of LPS, but the other half received sterile saline. These 10 animals served as controls, and were treated subsequently as follows: The former group of 5 LPS-injected chickens were challenged with 5 mls. of sterile (Seitz-filtered) normal chicken serum per Kgm., whilst the saline-injected animals were given 5 mls. per Kgm. of undiluted specific hyperimmune serum.

All the injected animals were bled after the last injections as follows: 15, 30, 60 minutes: 2, 4, 6, 24, 48 and 72 hours. The blood samples taken at these times were assayed for changes enumerated above.

LPS Injection Experiments with 'Immune' chickens : Animals from the first and second group experiments (that is, those given either a single dose (SD) or multiple injections of LPS) were left for between 28 to 30 days after the end of the experiments. It was considered that after this 'resting period', all the animals would have completely recovered haematologically; they would also be actively 'immune' or primed. They were then to be re-injected, either with the same S.D. of LPS or with multiple injections of LPS, as outlined above. Blood samples were to be treated as described above.

Post-mortem Examination : Some of the animals that had received multiple LPS injections, and their controls, were killed at the end of /

of the experiments, and the spleen and liver were removed and weighed. Individual chickens were again re-weighed.

RESULTS

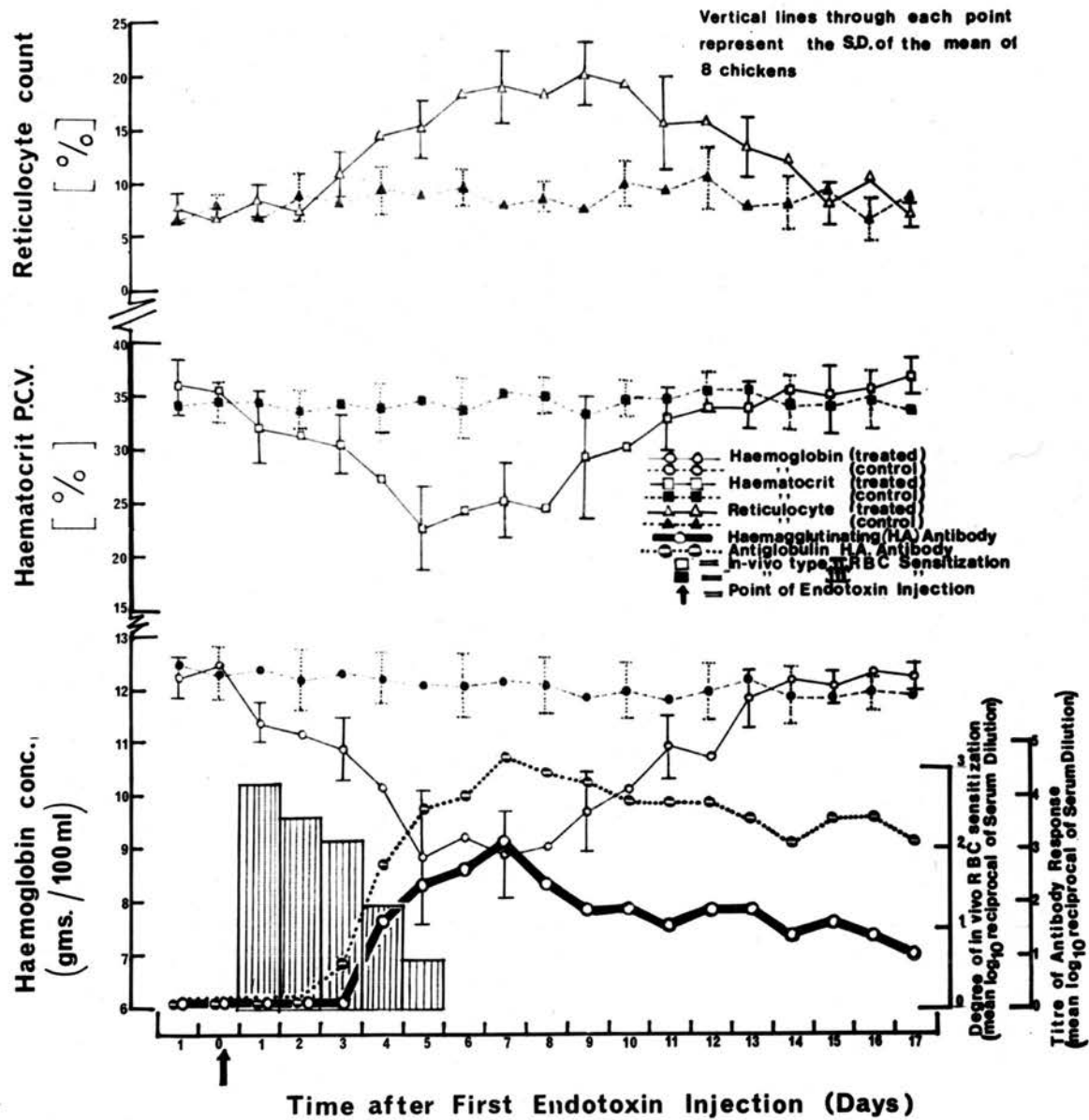
(1) Haematological and serological Responses to a Single Large Intravenous (i/v) Injection of LPS:

The responses in a group of chickens, each given a single, large dose of LPS (40 mgm. per Kgm.) i/v, are illustrated in Fig.47. Varying degrees of moderate reticulocytosis, not exceeding 15 per cent, with corresponding decreases in the haemoglobin concentrations (25 to 28 per cent of original values) and packed red cell volumes (27 to 30 per cent), were observed from the fourth to the ninth day after the injections of LPS. The time of haemolytic crisis coincided with the logarithmic increases in the antibody response (range of haemagglutinating antibody titre was between 1:160 and 1:1280). Fig.47 also shows that the onset of rapid elimination of sensitized erythrocytes coincided with the first appearance of the complete haemagglutinins. The antiglobulin haemagglutinins, however, appeared earlier and persisted longer at higher levels than the complete haemagglutinins.

The animals started to recover haematologically by the tenth day after administration of LPS, and were all normal when the experiments were terminated at the end of 17 days after injections.

Once the animals have had their erythrocytes sensitized in-vivo with the S.D. of LPS, polysaccharide coated erythrocytes could be demonstrated in the circulation for 4 or 5 days when they suddenly disappeared. ⁵¹Cr-labelling studies have earlier /

FIGURE 47. RESPONSE to a LARGE SINGLE DOSE of ENDOTOXIN



earlier shown that the sensitized erythrocytes have greatly shortened survival time (Chapter V).

These large doses of LPS did not elicit any obvious clinical signs.

No such changes were observed in control chickens which remained normal.

(2) Responses to Multiple Injections of LPS:

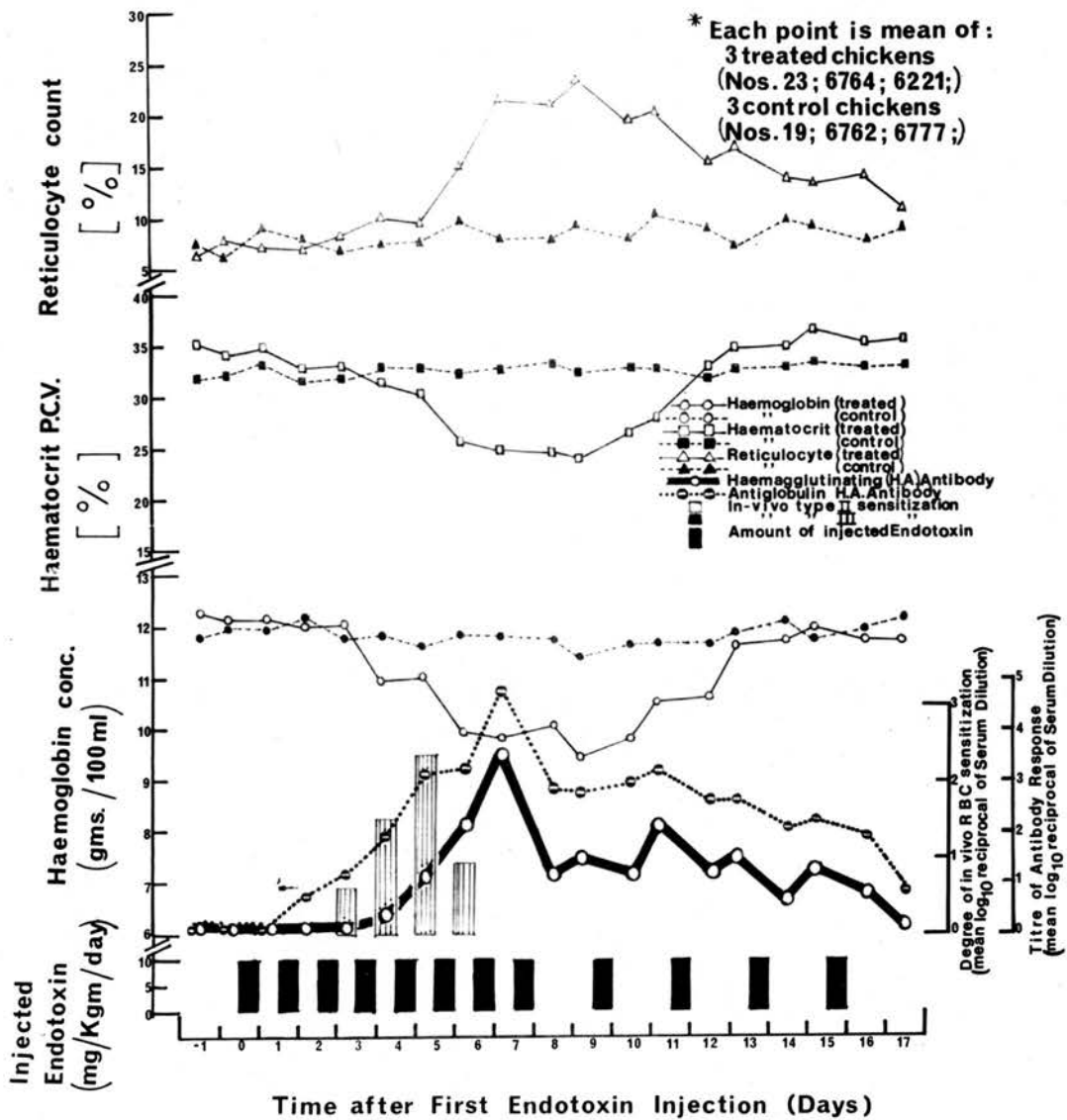
In contrast to the degree and pattern of the haemolytic response produced after single injections of LPS, repeated injections led to more striking haemolytic changes. The degree and pattern of the haemolytic changes were closely related to the quantities of the LPS injected, and the results of these studies are shown in Figs.48 - 50.

(i) Response to Graded Multiple Doses of LPS in Normal (Non-immune)

Chickens: Three groups of chickens received i/v various concentrations of LPS in the experimental protocol outlined in the test.

The first group of chickens, which received 10 mgm. of LPS per Kgm., daily for 7 days and on alternate days for another 4 days, developed moderately severe haemolytic episodes between the sixth and tenth days after the first injection (Fig.48). These animals also developed mild reticulocytosis (15 per cent increase), and corresponding decreases in the haemoglobin and haematocrit levels between the same period after the first injection. The suggestion that an active immune response is intimately involved in the mechanism of the haemolytic syndrome is supported by the finding that the complete, agglutinating antibodies appeared and persisted during this critical period. The incomplete, anti-globulin/

FIGURE 48. Effect of Multiple Injections of Endotoxin in the Chicken *



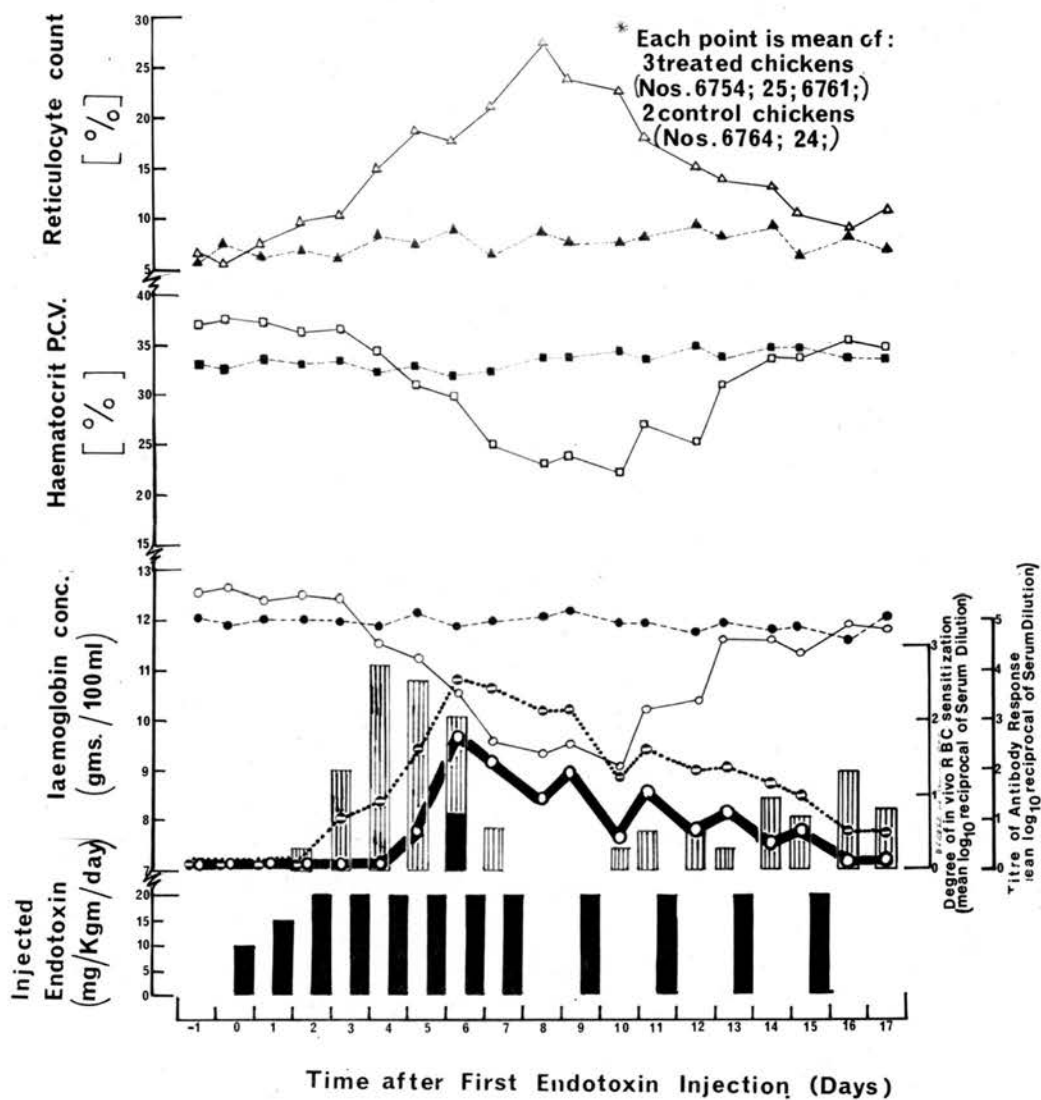
antibodies, which appear as early as the second day after LPS injection, do not seem to affect appreciably, the disappearance rates of the sensitized erythrocytes.

Another striking finding was that in all the animals the haemolytic process appeared to be self-limiting. Despite continued injections of LPS the reticulocyte counts decreased, and haemoglobin and the haematocrit levels increased during the last 7 days of observations. It was of great interest that it was found impossible to demonstrate sensitization of erythrocyte after 7 days, despite the repeated injection of LPS.

The second group of chickens which received larger doses than above — 20 mg. per Kgm. repeatedly — also showed increased disappearance of erythrocytes from the circulation (Fig.49). In this group of animals, reticulocytosis first appeared 4 to 6 days after injections of LPS were begun, and maximal reticulocyte count of 22 to 27 per cent (in contrast to the 6 to 9 per cent obtained for the control animals) occurred subsequently between the eighth and eleventh days after first injection of LPS. Haemoglobin concentrations, as well as the haematocrit levels, were also correspondingly decreased; the mean maximal changes being the reduction from 12.5 to 9 gms. per cent, and 37 to 22 per cent respectively.

The self-limiting nature of the haemolytic process, and the coincidence of the point of increased erythrocyte destruction with the appearance of the haemagglutinating antibodies, were again observed. However, the degree of induced anaemia, the amount of antibody engendered, and the general pattern of the haemolytic curves, differed from the first group of chickens. For example, partial in-vivo erythrocyte sensitization could be induced again after the initial/

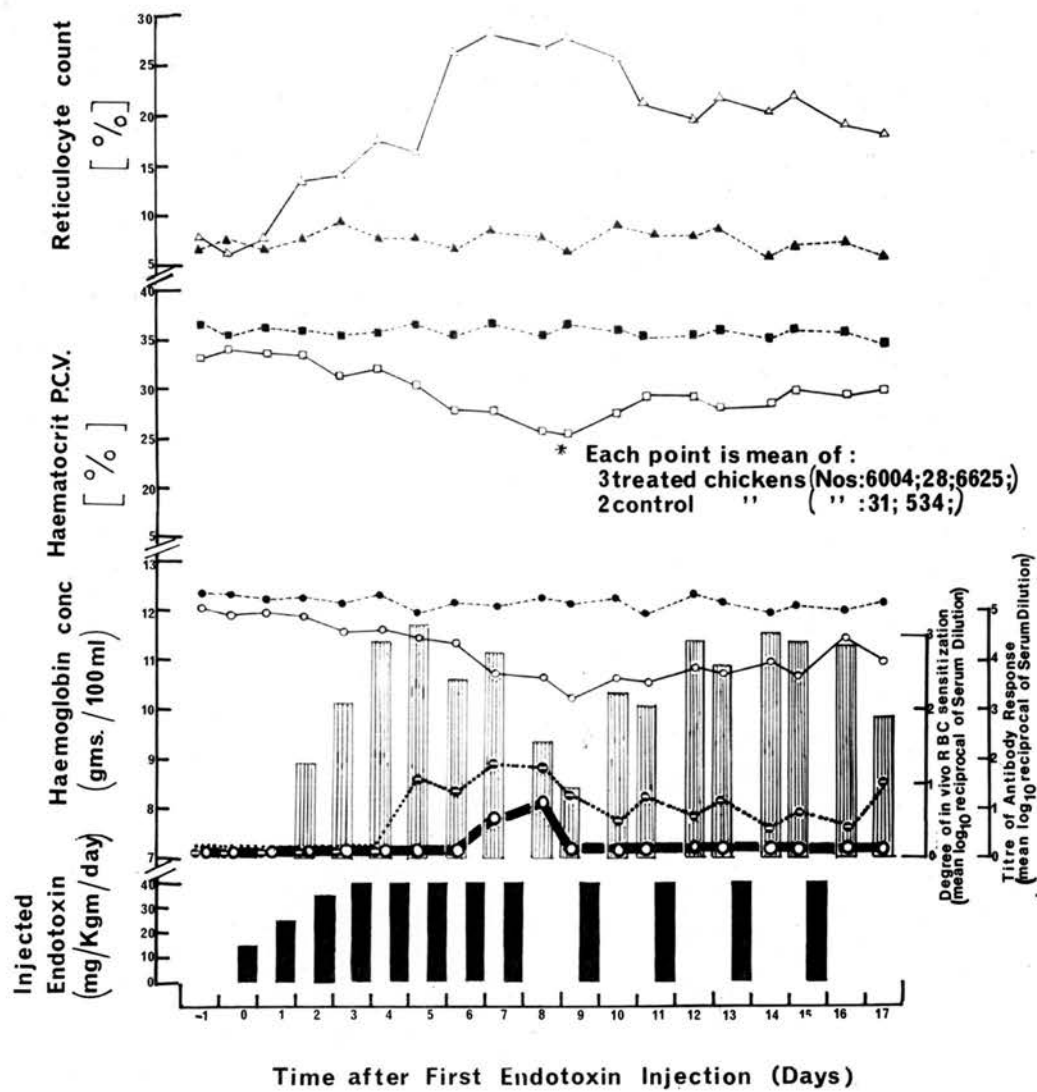
FIGURE 49. Effect of Multiple Injections of Endotoxin in the Chicken *



initial disappearance of the sensitized cells, and Type III sensitized cells could be detected at the point of highest level of specific serum antibody, even though, generally, a lower level of antibody was actively produced. Of great interest, however, was the observation that subsequent re-sensitization of erythrocytes in-vivo did not seem to have resulted in corresponding decreases in haematologic levels. Instead, haematologic recovery proceeded as if no sensitized erythrocytes existed in the circulation.

When still larger doses of LPS (40 mg. per Kgm.) were repeatedly administered, rather surprising results were obtained (Fig.50). The haemolytic crisis was much less severe, and sometimes the haematologic values were only slightly below normal levels. Thus, in spite of maximal degree of Type II erythrocyte sensitization induced by these large doses of LPS, the haemoglobin and haematocrit levels, on average, only fell from 12 to 10 gms. per cent, and 33 to 25 per cent respectively at the lowest haematologic levels. Interestingly however, reticulocytosis was highest in this group of animals. Maximal counts of up to 30 per cent were obtained in some of these chickens, 7 to 9 days after the first injection of LPS. Complete haemagglutinins could not be detected in this group of animals (Fig.50), and significantly, in-vivo Type III sensitization was not observed. However, the same self-limiting and recovery features of the haemolytic changes observed in the other groups were also detected with this group of animals. The minimal haemolytic changes tended to abate despite repeated administration of large doses of LPS, but in contrast to the other groups of injected chickens, their haematologic values had not fully returned to normal levels at the time of termination of experiments.

FIGURE 50. **Effect of Multiple Injections of Endotoxin in the Chicken ***



A most significant finding in all the groups of animal was the observation that the haemolytic response occurred several days — between 4 and 6 days — after the first injection of LPS. This strongly indicated that antibody response may be involved in the mechanism of the haemolytic episode.

In all these experiments, simultaneous control studies were conducted, and the results, shown together with those of the treated animals in Figs.47 - 50, demonstrate the absence of any significant changes in animals injected with only sterile saline. Therefore, bleeding per se did not affect the values obtained for the various haematologic parameters.

(ii) Effects of Repeated i/v Injections of LPS in 'Immune' Chickens:

The same graded dose range of LPS was given to the groups of chickens which had earlier received either a single, large dose or multiple injections of LPS one month after they recovered from the initial experiments. The reticulocyte, haemoglobin and haematocrit levels in such animals were invariably normal after one month's rest from injections.

The observations in these chickens were similar in both the pattern and degree of the haemolytic syndrome to those observed when normal animals were used, with the following significant exceptions:

(a) The anaemia appeared much earlier than when the chickens were in the non-immune state. It persisted longer, and was also more profound in intensity in all the injected groups, except those animals that had received multiple doses of LPS (40 mgm. per Kgm.), which still showed minimal haemolytic changes.

(b) Much higher degree of in-vivo erythrocyte sensitization was detected/

detected, and in contrast to the previously non-immune chickens, both Types II and III erythrocyte sensitization were detected. These types of sensitization were, however, restricted to the first two groups of chickens which had received multiple injections of either 10 or 20 mg. of LPS per Kgm. No type III sensitized cells were detected in the third group of chickens which had received 40 mg. per Kgm. multiple injections of LPS.

Persistence of sensitized erythrocytes in these multiple-injected animals was restricted to the Type II sensitized cells, and this was observed only in groups of animals which had received multiple injections of either 20 or 40 mg. of LPS per Kgm.

(c) As with the previously non-immune animals, there was no visible haemoglobinaemia after administration of the first dose of LPS in any of the 'immune' chickens, even after injection of 40 mg. of LPS per Kgm.

(iii) Significance of Splenic Activity: Mild splenomegaly was found uniformly only in the groups of animals that had received multiple doses of LPS. Saline-injected control chickens had a mean splenic weight of 0.84 gms. per Kgm. body weight, whereas the animals that received repeated doses of LPS had a mean splenic weight of 1.3 gms. per Kgm. body weight. There was no increase, however, in the mean splenic weight of the animals that had received single, large LPS injections.

All the animals were killed after all the experimental observations had been completed.

(3) Effect of Passively Administered Antibody on Experimentally-Induced in-vivo Erythrocyte Sensitization:

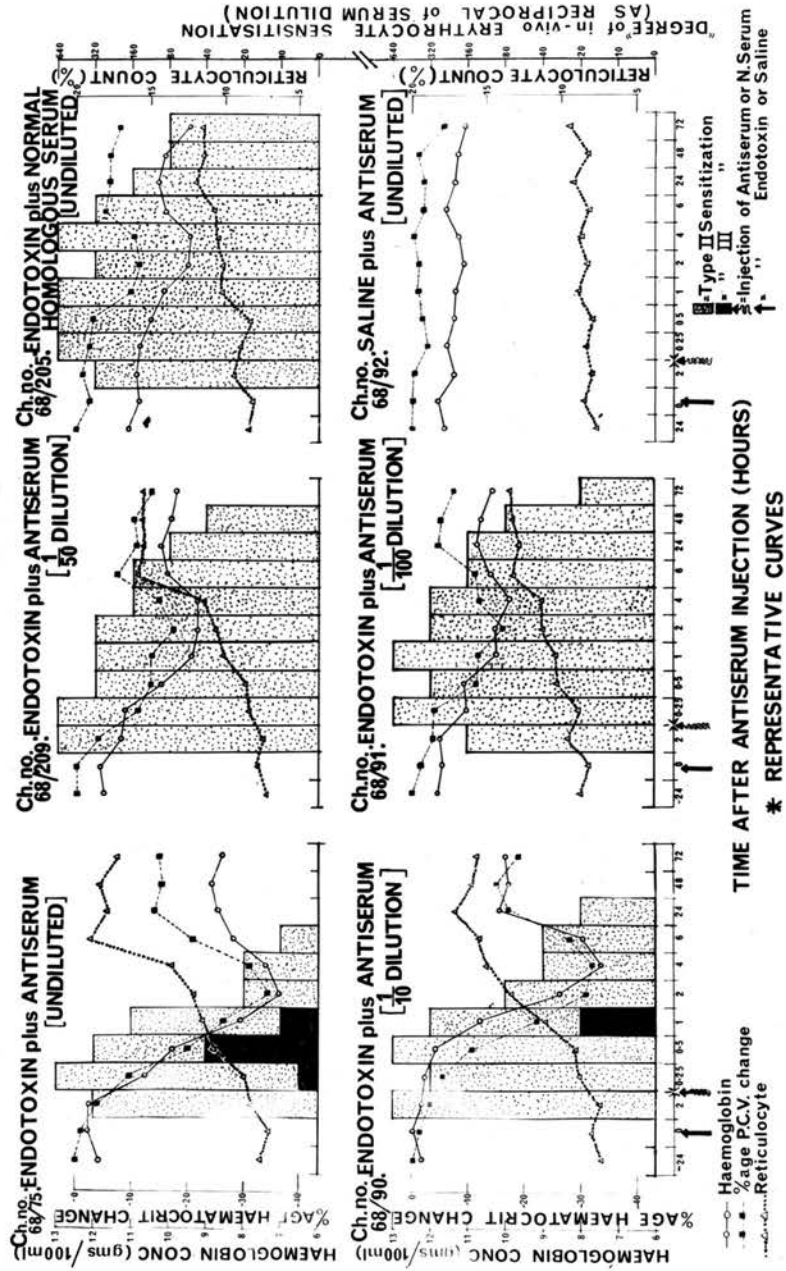
From the results so far obtained, the haemolytic process/

process occurs approximately a week after the first injection of LPS, and was more profound in animals that had received multiple doses of LPS. This suggested that antibody may be involved. Therefore the effect of passively injected specific antibody to LPS after prior LPS administration, was investigated. The results are depicted in Figs. 51 and 52.

(i) Induction of Anaemia using Homologous Antibody in LPS-Injected

Chickens: As shown above, i/v injection of an LPS dose of 40 mg. per Kgm. into chickens induces sensitization of erythrocytes in-vivo. Two hours after this injection, when the animals had completely recovered from any signs of distress and shock, and when maximum degree of erythrocyte sensitization could be detected, they were challenged with various doses of homologous antiserum. The results obtained from these experiments, shown in Fig. 51, were highly significant. They demonstrate clearly that a significant percentage of the animals' total body erythrocyte volume is sequestered following the injection of specific antibody. As early as one hour after the i/v injection of antibody, significant decline in the haemoglobin and haematocrit levels were observed in all the LPS-injected animals. At the same time, distinct rises in the reticulocyte counts were obtained, the mean peak reticulocytosis being observed 6 hours after the injection of antibody. Typically, maximum decreases in haematologic values were obtained 2 hours after antibody injection. For example, in chicken number 68/75 which had received the highest concentration (undiluted) of antiserum, the decline in the haematologic values started as early as 15 minutes after antibody administration, and by the end of 2 hours afterwards, the animal had lost approximately over 30 per cent of its total circulating/

FIGURE 51. EFFECT of PASSIVE ANTIBODY on CLEARANCE of in-vivo SENSITISED ERYTHROCYTES *



circulating number of erythrocytes.

Another important observation in these studies was the rapidity with which the haematologic recovery phase followed the peak of the haemolytic episode. The rapid recovery phase was subsequently found to be due to the sudden appearance of large numbers of immature erythrocytes (reticulocytes) in the circulation. This was, however, not surprising, in view of the known ability of LPS to stimulate erythropoiesis in animals (Smith, 1964; Fruhman, 1966; Boggs, 1966; Boggs, Chervenick, Marsh, Cartwright and Wintrobe, 1968).

Significantly, erythrocytes from animals challenged with the highest concentrations of antibody were also found to be sensitized with the antigen-antibody complex (Type III sensitization) — see chicken numbers 68/75 and 68/90 in Fig.51. Moreover, these particular animals showed the most profound of the haemolytic crisis.

Inspection of the various curves in Fig.51 confirms earlier suggestion that there is an intimate relationship between the phenomena of in-vivo erythrocyte sensitization and induction of haemolytic anaemia after LPS injection (Chapter V). The rate of reduction in the 'degree' of in-vivo sensitization was found to be closely related to the degree of induced anaemia. For example, chicken number 68/75, which had cleared all its sensitized cells from the circulation by the end of 24 hours after antibody injection, showed the severest anaemia. In contrast, in chicken numbers 68/205 and 68/91, where sensitized cells could still be demonstrated even after 72 hours, the induced anaemia was only minimal.

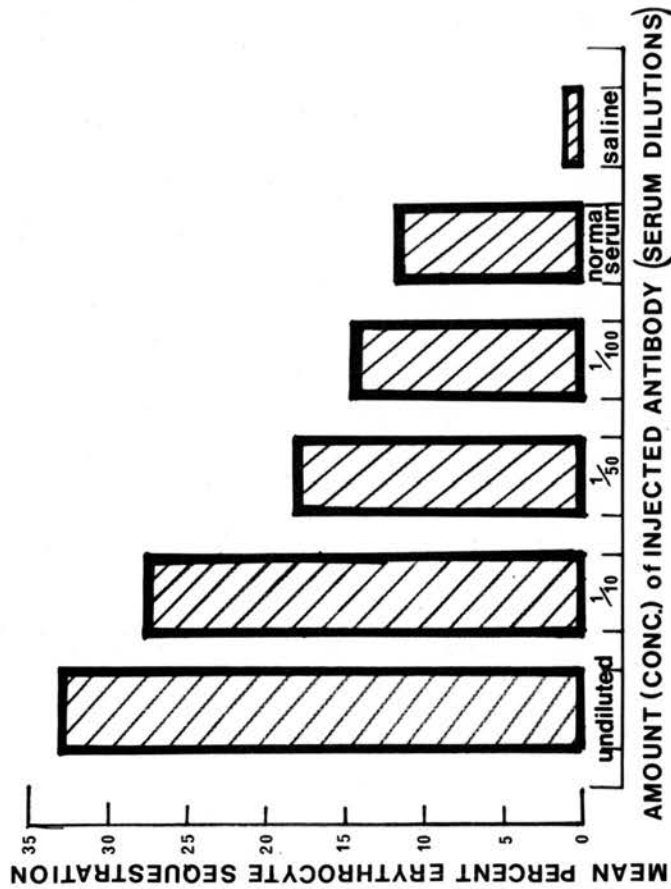
Control chickens (for example, number 68/92) which had/

had been injected with sterile saline and undiluted antiserum, showed no anaemia.

(ii) Relationship between the level of Specific Antibody and the Number of Sensitized Erythrocytes Sequestered: The results recorded in Fig.51 showed that passively administered specific antibodies augment or accelerate the haemolytic process that occurs after injection of LPS. Furthermore, it was found that the percentage of sensitized erythrocytes sequestered within a specific time was directly related to the concentration of the injected antibody. This relationship is illustrated in Fig.52. In response to decreasing concentrations of antibody, a progressive decrease in the volume of sequestered erythrocytes was observed in the animals 2 hours following antibody challenge. A clear dose-response relationship between the concentration of antibodies in the circulation and the percentage volume of sequestered sensitized erythrocytes at 2 hours, thus existed. More than 30 per cent of the animals' sensitized erythrocytes could therefore be destroyed by challenging LPS-injected animals with a potent, high-titred (undiluted) homologous antiserum. In contrast, only about a third of this volume was sequestered, using LPS alone. Furthermore, an insignificant amount of erythrocytes were destroyed in animals where there had been no in-vivo sensitization (control chickens given sterile saline), even with the subsequent injection of the highest concentration of homologous antiserum.

(iii) Clinical manifestations: Clinical symptoms after LPS and homologous antibody injections closely resembled those of the terminal stages of chickens infected with acute fowl typhoid. These were salivation, dyspnoea, cyanosis, incoordination, diarrhoea and/

FIGURE 52. RELATIONSHIP BETWEEN the AMOUNTS of PASSIVE ANTIBODY and SENSITIZED R.B.C.s SEQUESTERED



a) ABSCISSA shows the mean percentage of sensitised erythrocyte sequestration measured at 2 hours after injection of homologous antiserum - and in the case of controls, normal serum or sterile saline

b) Percent erythrocyte sequestration = Percentage Haematocrit change

and pyrexia. It was considered of great significance that all the typical clinical signs appeared only after subsequent challenge of LPS-injected chickens with anti-LPS antibody.

DISCUSSION

The possibility that adsorption of bacterial antigen(s) and subsequent interaction of antibody and antigen(s) at the erythrocyte surface may occur in-vivo, and if so, whether this can produce erythrocyte destruction, has in the past led many attempts to experimentally induce in-vivo erythrocyte sensitization by injection of various bacterial polysaccharides. Thus, although Ceppellini and De Gregorio (1953) had earlier been unable to induce in-vivo sensitization in rabbits by injection of Vi-polysaccharide, De Gregorio (1955) was later able to achieve this in guinea-pigs, but only after injection of large amounts (10 mg.) of the polysaccharide. That in-vivo sensitization was possible, but only after the administration of relatively large amounts of bacterial polysaccharide is confirmed by the observations of Boyden (1953) in guinea-pigs and Buxton (1959a) and Springer and Horton (1964) in chickens.

It is of interest that none of the above authors reported the occurrence of anaemia after these large, single injections, although the possibility that this occurred should not be ruled out. The present results suggest that anaemia might well have developed following injection. In support of this assumption is the finding that after intravenous injection of endotoxin there is a decrease in the haematocrit level in the chicken (Jordan and Hinshaw, 1964) and the monkey (Hinshaw, Jordan and Vick, 1961). These/

These authors, however, did not undertake any investigation of surface changes on the erythrocytes, but the recent reports that certain experimental animals given endotoxin either have decreased erythrocyte life-span (Cartwright, 1966; Sherman, Krumhaar, Tanaka, Bennet and Maloney, 1966) or develop gross bilirubinaemia (Eddington and Kampschmidt, 1968) would indicate the ability of endotoxin to induce haemolysis under their experimental conditions. Although the above results hinted at an intimate relationship between the erythrocyte surface sensitizing properties of endotoxin and the various haematologic manifestations caused by endotoxins, the precise mechanism of these changes has hitherto not been clearly demonstrated. The present results have clearly shown that, in fact, the anaemia induced by endotoxin injection is the direct consequence of such erythrocyte surface sensitization.

Haemolytic anaemias are generally accompanied by abnormalities in the red cell or in the host (Chapter I), and it was also demonstrated in previous chapters that specific immune alteration of the erythrocyte surface is an essential pre-condition in the pathogenesis of the haemolytic anaemia associated with acute S.gallinarum infection. The present results confirm this finding by clearly demonstrating that chickens develop relatively severe haemolytic anaemia after either single or multiple injections of endotoxin derived from S.gallinarum, and that this anaemia is related to an abnormality of the erythrocyte; that is, sensitization. Furthermore, following endotoxin injection, circulating specific antibody is demonstrable and the period of haemolytic crisis coincides with the disappearance of these sensitized erythrocytes from the circulation. The significance of specifically reacting antibody/

antibody in this haemolytic syndrome was further indicated by passive injections of varying amounts of hyperimmune serum following induction of in-vivo sensitization by endotoxin injection. Varying degree of anaemia, from moderate to severe, depending on the dose of the antibody, followed within a short time of the injection, together with clinical and pathological signs highly reminiscent of the natural disease process. These findings, coupled with determinations of decreased erythrocyte survival in endotoxin-injected chickens (Chapter V), provide strong evidence demonstrating that this induced anaemia is haemolytic and may be due to the attachment in-vivo of endotoxin to erythrocytes, followed by their rapid sequestration in the presence of anti-endotoxin antibody, as previously suggested to account for this anaemia (Chapter V) and also earlier, postulated for the induction of anaemia of fowl typhoid (Chapter III).

This mechanism may also explain the more severe and much earlier appearance of the haemolytic episode observed in the immune chickens after re-inoculation with endotoxin. Significantly in this particular group of animals, the erythrocytes were found to ~~be~~ exhibit Type III sensitization after re-injection with endotoxin.

However, there is the alternative possibility that the haemolytic response may be due to an abnormal increase in the erythrophagocytic activity per se of the reticuloendothelial system (RES), as has been postulated by Ho and Kass (1958) to account for the haemolytic anaemia developed in rabbits after endotoxin injections. Ho and Kass observed that after prolonged intravenous injection of S. typhi endotoxin into normal rabbits, a mild to severe haemolytic anaemia developed. A more marked anaemia developed in immunized rabbits/

rabbits which were subjected to repeated inoculations of endotoxin, and the haemolytic changes reached their peak during the second week after the first injection. This suggested that antibody may be involved, but their failure to demonstrate the presence of in-vivo erythrocyte sensitization led them to suggest that the development of the anaemia was due to increased activity of the RES. There was, however, enough evidence of their results to indicate that the anaemia they observed was not the result of an as yet unexplained activation of the RES. For instance, the anaemia was not well correlated with the size of the spleen, the largest spleen was found only in an hyperimmune rabbit that was no longer anaemic. Furthermore, splenectomy did not appear to eliminate the haemolytic response to endotoxin. On the other hand, the antibody to endotoxin appeared during the period of haemolytic crisis, and the possibility that cells coated with endotoxin in-vivo were then removed rapidly from the circulation of animals, and hence were not detectable peripherally, cannot be excluded. Such an occurrence might even explain the haemoglobunaemia they observed in the immune animal shortly after injection of a large dose of endotoxin.

Nevertheless, even though RES function was not directly investigated, for example by carbon clearance, the anaemia herein described is not well correlated with the size of the spleen and liver. Thus, a mild splenomegaly was observed only in chickens given multiple injections of endotoxin, whereas single injections of endotoxin, although this similarly induced a severe anaemia, were not accompanied by any significant increases in either the spleen or liver sizes. Moreover, previous studies (Chapter V) have shown that intravenous injection of a large dose (40 mgm. per Kgm. body weight)/

weight) of endotoxin in the chicken — 6 or 24 hours prior to transfusion of ^{51}Cr labelled erythrocytes — resulted in a slight decrease in the concentration of radioactivity, but only during the first 24 hours; beyond this 24 hour period, the survival was normal. In contrast, following administration of identical materials in the reverse order, the erythrocyte life-span was found to be markedly reduced (Fig.38). These observations militate against a generalised stimulation of the RES per se being the cause of this anaemia, and suggest that the increase in weight of the spleen in multiple endotoxin-injected animals may be due to the deposition of material from the erythrocyte destruction.

On the other hand, the anaemia described here is well correlated with the time of in-vivo sensitization and the onset of active immune response. For instance, it was found that in-vivo sensitization could be detected throughout the early part of the haemolytic process, particularly those given single injections, but the major haemolytic response occurred only approximately one week after the injection of single or multiple doses of endotoxin, which strongly indicated that the anaemia induced is antibody-mediated.

The role of antibody in the production of this haemolytic episode was subsequently amply demonstrated by the experiments which consisted of passive injections of specific anti-endotoxin antibody to endotoxin-injected recipients with in-vivo sensitized erythrocytes. The results of these experiments show that such a procedure greatly accelerated the rate of erythrocyte destruction and also augmented the reticulocyte response (Fig.51). The observation that the total percentage of erythrocyte sequestered — hence the severity and degree of the induced immunohaemolytic anaemia/

anaemia — was dependent on the potency or titre of the administered homologous antibody (Fig.52) offered another convincing evidence in support of the contention that specific immune antibodies are directly involved in the destruction of endotoxin-injured erythrocytes.

In the present experiments, the haemolytic process was found to be self-limiting, the haemolysis tending to abate despite repeated administration of endotoxin; and the finding that the severity of the induced haemolytic anaemia was markedly decreased when very large doses (40 mgm. per Kgm. of body weight) of endotoxin were injected daily (Fig.50) is probably explained by the unique **ability of certain polysaccharides to induce immunological paralysis** in a variety of adult animal hosts (Britton and Möller, 1965; 1968; Dresser and Mitchison, 1968; Britton, 1969). The almost complete absence of de novo formation of haemagglutinating antibodies in this group of animals (Fig.50) attest to the above suggestion. It is also quite likely that it was this immunologic unresponsiveness which was the significant factor in the failure to induce any significant degree of antibody-mediated destruction of erythrocytes in this group of chickens.

The present results have demonstrated, in general, that the release of endotoxin during the bacteraemic phase of acute S.gallinarum infection plays a primary role in the pathogenesis of the accompanying haemolytic anaemia. The immunological basis of the overall pathogenesis of the disease is also indicated by the finding that clinical signs, closely resembling those of the terminal stages of acute fowl typhoid, appeared only after animals, previously injected with large doses of endotoxin, were challenged intravenously with anti-endotoxin antibodies.

These/

These findings show that Fowl Typhoid offers an excellent experimental model for studies of the anaemia of gram-negative bacterial infections in general. Its advantage is due to the extreme tolerance of the chicken to endotoxins which resist many times the dose lethal for most commonly used laboratory animals (Gilbert, 1960; Kuida et al., 1961; Berczi, et al., 1966). This tolerance enables large quantities of endotoxin to be employed experimentally, and also in the natural disease, probably allows the systemic build up of similar large amounts of endotoxin, with the result that heavy and widespread erythrocyte sensitization occurs, thus permitting their serological demonstration. Furthermore, none of the common laboratory animals can offer the combination of extreme susceptibility to a natural host-specific infection, with the phenomena of in-vivo erythrocyte sensitization and clinical haemolytic anaemia, together with unique refractoriness to the lethal effects of experimentally administered endotoxin.

CHAPTER VIIITHE SIGNIFICANCE OF ANAEMIA IN THE PATHOGENESIS
OF S. GALLINARUM INFECTIONINTRODUCTION

It has been stressed in previous chapters that one of the main pathological features of acute fowl typhoid is the haemolytic anaemia. During this infection, a massive destruction of the erythrocytes occurs (Chapter II), with the result that the fatally infected animal loses more than 70 per cent of its original total circulating number of erythrocytes (Chapter V). It is probable that the effects of such a striking haematologic change may alone be extremely significant in the overall pathogenesis of the disease. It was also shown in Chapter V that the main organs responsible for the final destruction of most of the sensitized erythrocytes during fowl typhoid belonged to the reticulo-endothelial system (RES), namely the spleen and liver. It is therefore also possible that the phagocytosis of the altered erythrocytes by the RES may lead to impairment of other important RES functions. In view of the general belief that bacterial endotoxin (LPS) is the chief moiety responsible for the pathogenesis of gram-negative bacterial infections (Bennet, 1948; Neva and Morgan, 1950; Spink and Anderson, 1954; King and Wood, 1958; Rapaport et al., 1964; Griesman et al., 1964; 1969; Penhale, 1965; Heddleston, Rebers and Ritchie, 1966; Riedler and Scheitlin, 1969), and the finding that damage to the RES increases host susceptibility to endotoxin (Formal, Noyes and Schneider, 1960; Cooper and Stuart, 1961; Box and Briggs, 1961; Suter, 1964; Farrar and Magnani, 1964), the possibility that the anaemia of fowl typhoid may/

may contribute significantly to the pathogenesis of this disease, also by an effect on the RES, becomes apparent.

Investigations in previous chapters have emphasised the ubiquity of endotoxin in the mediation of many of the pathologic and patho-physiologic effects in chickens infected with S. gallinarum. Earlier studies of this disease by Buxton and Davies (1963) have shown wide variations of endotoxin in the reticulo-endothelial organs after infection which could not be related to the lethal outcome of this disease. However, evidence that a major proportion of endotoxin injected into experimental animals localises within the tissues of the reticulo-endothelial system, (Braude et al., 1955; Cremer and Watson, 1957; Carey et al., 1958; Chedid, Skarnes and Parant, 1963; Golub, Groschel and Nowotny, 1967; 1968), and certain experimental procedures altering the RES function may have profound effects on the animals' susceptibility to endotoxin (Good and Thomas, 1952; Suter, Ullman and Hoffman, 1958; Benacerraf, Thorbecke and Jacoby, 1959; Connor and Kass, 1961), suggests that a physiologic lesion of the RES plays an important role in the overall reaction of the animal to endotoxin.

The possibility therefore existed that the anaemia of acute fowl typhoid may be an important pathogenic factor for several reasons. These possibilities were examined in the following studies, where it was attempted to determine the significance and probable role of anaemia in the pathogenesis of the disease.

MATERIALS AND METHODS

Experimental Animals: Approximately 200 ten-week old White-Link chickens of both sexes were used in these experiments. The animals, which/

which were obtained in 6 batches of about 30 animals per batch, were each used for a corresponding single set of experiments (vide infra). Each group of animals was housed, fed and treated under the same conditions; and each animal was weighed on arrival and re-weighed on the day of experiment.

Haematocrit determination and Reticulocyte count: These were carried out as described previously (Chapter II).

Preparation of Endotoxin (LPS): Large quantities of purified LPS were prepared by the method described in Chapter III. After freeze-drying the material was accurately weighed, batched, vacuum-sealed and stored until required, when it was reconstituted in sterile saline.

Estimation of LD_{50} of LPS: The dose-effect of the experiments was evaluated by estimating the 50 percent mortality dose. This was done by 2 methods, and the mean value then found.

(i) Doses of LPS injected were plotted against Log_{10} percent mortality on Probability paper for linearity.

(ii) Method described by Reed and Muench (1938).

EXPERIMENTAL DESIGN

The chickens, consisting of 6 groups of approximately 30 animals per group, were treated as follows:

Group I:- Anaemia (induced by chronic bleeding); no LPS injected.

Group II:- Injection of LPS only.

Group III:- Oligocythaemic anaemia (induced by withdrawal of a large amount of erythrocytes), followed by injection of LPS.

Group IV:- ? Functionally impaired RES (induced by erythrophagocytosis of heterologous-rabbit-erythrocytes), followed by LPS injection.

Group/

Group V:- ? Functionally impaired RES (induced by erythrophagocytosis of in-vivo sensitized chicken erythrocytes), followed by LPS injection.

Group VI:- ? Functionally impaired RES (induced by erythrophagocytosis of in-vivo chemically altered autologous cells), with consequent development of haemolytic anaemia; these were also injected with LPS.

The various doses of LPS are given for each group in Table 23.

Experimental Protocol:

The various doses of LPS, the regimen of daily bleeding and the procedures for producing anaemia, and the induction of probable impairment of the reticulo-endothelial function by erythrophagocytosis, were all determined after preliminary experiments.

Group I: Preliminary experiments showed that up to between 10 and 15 mls. of blood could be withdrawn daily from each animal, for many days, with apparently no ill-effects.

(a) Regimen and Procedure of Bleeding: A small blood sample (less than 1 ml.) was withdrawn into Sequestrene tubes for haematocrit and reticulocyte level determinations. Then, from the other wing vein, an accurate amount of blood was withdrawn into a calibrated syringe with a measured volume of isotonic heparin. The blood was centrifuged, and an equal volume of autologous plasma was re-transfused into the appropriate animals within 30 minutes.

(b) Estimation of Volume of Erythrocytes Removed per Day: With the retransfusion of plasma back into respective animals, and the progressive/

progressive decrease in the haematocrit levels as a result of the daily bleeding, the volume of erythrocytes present in the daily blood sample was considered the more relevant parameter for comparison.

The volume of erythrocytes removed per day (V_E) in a blood sample (V_B) with a known haematocrit (H_B), was estimated as follows:

$$V_E = \frac{V_B \times H_B}{100} \quad (\text{in mls.})$$

The control animals were bled as before, but the packed cells, suspended in a measured volume of autologous plasma, were transfused back into the respective animals.

Group II: This group of animals was divided into 4 sub-groups and injected i/v with varying doses of LPS without prior treatment. The purpose of these injections was to establish the normal lethal dose of LPS for comparison with the treated animals.

Group III: On the basis of the data from haematologic and radio-chromium-labelling studies (Chapters II and V) it was estimated that the apparent average percentage of erythrocytes destroyed at the time of death was approximately 50; it was nearly 70 percent if only the animals' original erythrocyte numbers were considered. Usually, on average, about 35 mls. of blood were removed from each animal, spread over 2 hours, in order to obtain a degree of anaemia comparable to that caused by infection. That this procedure of inducing acute blood-loss anaemia was relatively safe, is supported by the finding that removal of more than 50 percent of blood in a single bleeding from an adult chicken did not result in the development of an irreversible shock and death (Wirth and Kubasta, 1939). As a further precaution, /

precaution, the chickens were challenged with the LPS only after their respective sera had been re-transfused and then left to 'rest' for between 5 and 6 hours. The object was to keep to a minimum, any possible activation of the RES activity. In this group, blood was not collected with heparin anticoagulant because of its detoxifying effect on LPS toxicity in animals (Schultz and Becher, 1967; Margaretten, McKay and Phillips, 1967; Gupta and Reed, 1968). The procedure was therefore to take a small amount of blood in Sequestrene tubes for PCV and reticulocyte count, and then bleed the animal without anti-coagulant. Afterwards, the blood was allowed to clot, and the serum auto-transfused as quickly as possible.

In the control experiments for this group, blood was taken in heparinized tubes and erythrocytes separated by centrifugation; the cells were then retransfused, suspended in homologous normal chicken serum which had been made up to the original volume. The control animals also received the same doses of LPS as the test animals.

Group IV: Preliminary studies had shown that normal chickens possess high levels of natural rabbit haemagglutinins—mean values of 1:64. They also exhibited no adverse clinical signs of intravascular haemolysis after injection of 2 mls. of 50 percent suspension of rabbit erythrocytes in sterile saline, but more than 4 mls. of this suspension was fatal when injected intravenously.

After injection of rabbit erythrocytes, the animals were left for over 6 hours before being challenged with LPS, at which time it was considered that nearly, or all the heterologous cells, have been sequestered. This assumption was based on the finding that non-viable or heterologous erythrocytes are cleared in many hosts at/

at a rate of 0.2 mls. of cells per Kgm. per hour (Noyes, Bothwell and Finch, 1960; Bowman et al., 1961; Mollison, 1962).

Group V: Each animal in this group received 3 mls. of 50 percent suspension of in-vivo sensitized chicken erythrocytes in sterile saline, and 6 hours later they were challenged with LPS.

The controls for both groups, IV and V, consisted of i/v injection of either sterile saline or homologous normal chicken erythrocytes, before being injected with LPS. Those animals which received normal chicken cells were checked for the absence of any iso-haemagglutinins.

Group VI: The ability of phenylhydrazine hydrochloride to induce a non-immunologic haemolytic anaemia in a variety of species is well-known (Muirhead, Groves and Bryan, 1954; Lowy, Keighley, Borsook and Graybiel, 1959; Weed, Eber and Rothstein, 1961; Azen and Shilling, 1963; 1964). Preliminary experiments had shown that, on average, a subcutaneous dose of 50 mg. of phenylhydrazine hydrochloride (PHND-HCl), dissolved in distilled water, reduced the haematocrit of the chicken from a mean value of 34 to 19 percent within 24 hours.

Sub-groups of chicken were thus given various doses of LPS, 30 hours after the injection of the drug.

Control animals were given sterile distilled water subcutaneously in place of the PHND-HCl, before LPS was injected.

A common procedure for LPS injections in all these experiments was to administer relatively large doses at the beginning, and then follow with the smaller doses if the resulting mortality was high. Appropriate adjustments of the doses and the procedure of/

of injections were made accordingly, depending on the number of animals which dies after each dose of LPS.

RESULTS

(1) Clinical and Haematologic Responses of Chronic Erythrocyte Loss:

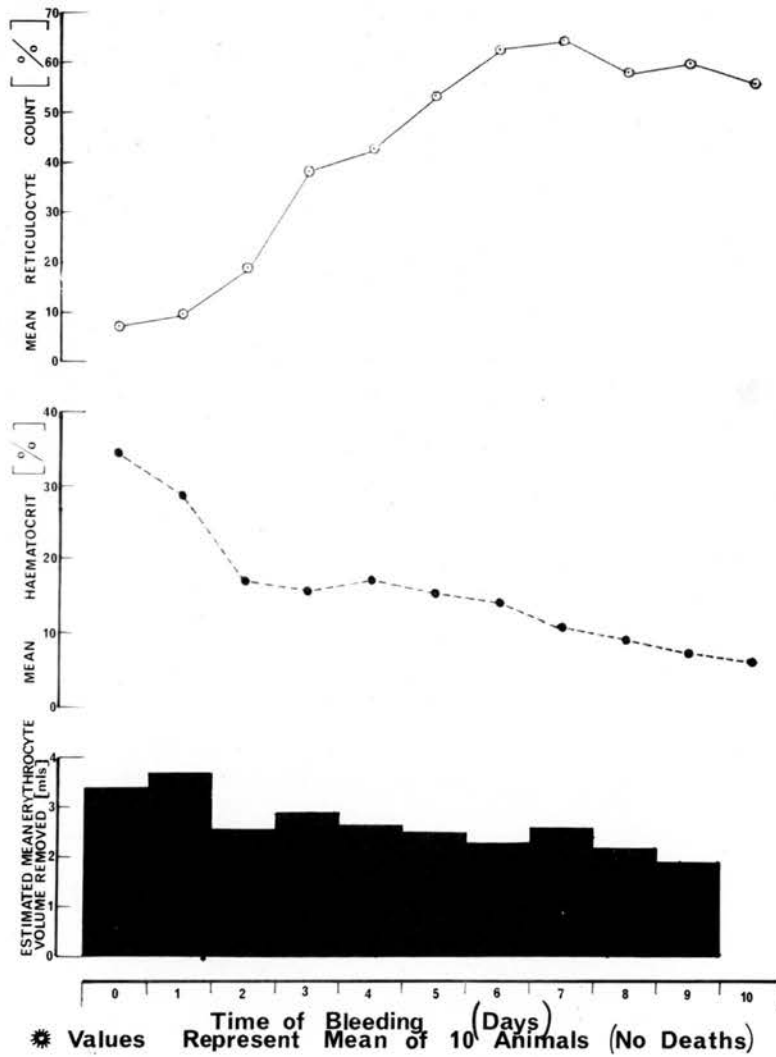
The significance of anaemia per se in the pathogenesis of a fowl typhoid was investigated by subjecting a number of animals to a regimen of daily bleeding with the object of producing a degree of anaemia comparable to that caused by S. gallinarum infection. Daily bleeding for more than a week resulted in the establishment of a severe chronic anaemia. The chickens showed the effects of anaemia, such as pallor and cyanosis of the comb and wattles. The other clinical signs were listlessness, ruffling of the feathers and difficult breathing.

As demonstrated in Fig. 53, there was a rapid decrease in haematocrit in the first two days when approximately 50 percent of the original erythrocyte volume was withdrawn. This period coincided with the onset of the increase in the reticulocyte count, and by the end of one week after the first bleeding, more than 60 percent of the total erythrocyte volume consisted of immature blood cells, mainly reticulocytes.

After the second day, subsequent decreases in the haematocrit level with bleeding, were small but gradual, in contrast to the massive but progressively rapid increase in the reticulocyte level during this period. This latter response, which may be due to increased haemopoietic activity, definitely accounted for the small decreases in the overall erythrocyte total numbers, in spite of repeated withdrawal of relatively large volumes of blood.

In/

FIGURE 53. **Effect of Chronic Bleeding on Haematocrit and Reticulocyte Values ***



In spite of the large amounts of blood withdrawn with consequent severe anaemia comparable to that found during fowl typhoid, none of the animals died. This indicated that the anaemia per se is not the chief lethal factor of infection.

There were no significant changes in the haematologic values for the control animals; furthermore, no adverse clinical signs were observed in this group of animals.

(2) Susceptibility of Chickens to *S. fallinarum* Endotoxin After Various Treatments:

Following the investigation of the effect of anaemia per se, it was decided to investigate the effect of anaemia in combination with LPS in the chicken. The effects of various treatments of chickens on their susceptibility to LPS are summarized in Table 23 and depicted in Fig. 54.

(i) The Effect of Acute Erythrocyte-Loss (Oligocythaemic) Anaemia on Susceptibility to LPS: The influence of acute erythrocyte loss on the susceptibility of the chicken to LPS was investigated by inducing an oligocythaemic anaemia by bleeding as above and subsequently challenging with LPS.

Inspection of Table 23 reveals that the removal of approximately 50 percent of blood, a procedure which produced a degree of anaemia comparable to that caused by *S. gallinarum* infection, did not appreciably increase susceptibility of chickens to LPS. With an LD₅₀ of 55 mg. per Kgm., they were equally susceptible to LPS as normal, untreated chickens (LD₅₀ of 56 mg. per Kgm.). The death rate and the cumulative mortality were also nearly the same in the bled and normal chickens, both being represented in/

in the high-dose region of Fig. 54.

(ii) The Effect of Transfusion of Heterologous Erythrocytes:

After demonstrating that there was no increase in susceptibility to LPS in chickens after acute erythrocyte loss, the effect of LPS in chickens which had cleared foreign erythrocytes from their circulation, and in which therefore RES activity may have been consequently reduced, was then examined.

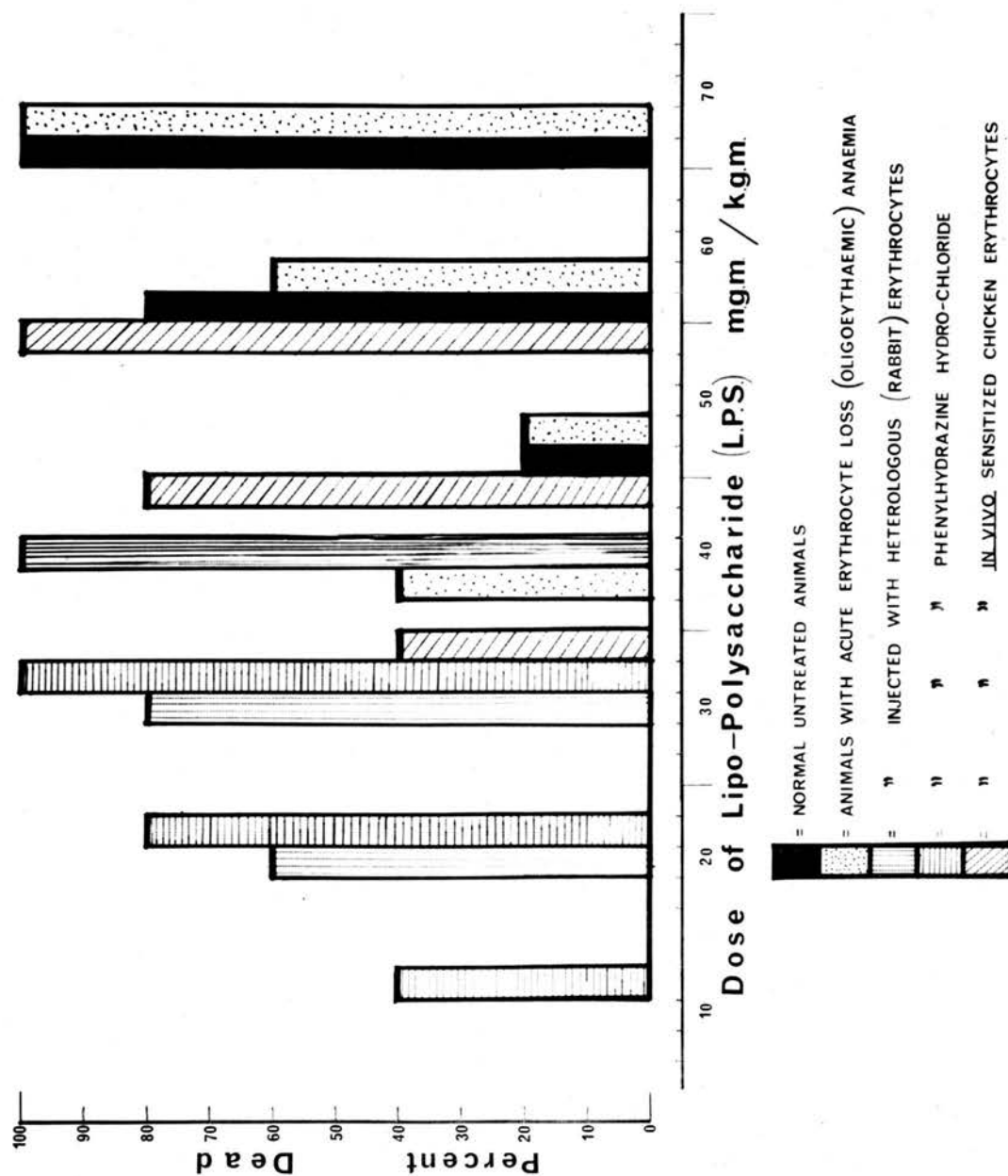
It was found that chickens inoculated with rabbit erythrocytes, and which had shown no clinical signs as a result of this injection, when challenged 6 hours later with LPS died at a much faster rate than either chickens pre-treated with homologous, normal erythrocytes or untreated animals. In the experiment summarised in Table 23, it is seen that the mortality rates in chickens injected with rabbit erythrocytes were 60 and 100 per cent, with doses of 20 and 40 mg. of LPS per Kgm. respectively. In contrast, there were no deaths until 50 mg. of LPS per Kgm. had been injected into normal, untreated chickens, and then only 1 of the 5 animals died.

The LD_{50} for the normal animals was estimated to be 56 mg. per Kgm. a dose more than twice the amount required to kill approximately the same percentage of animals pre-treated with rabbit erythrocytes (21.5 mg. per Kgm.).

None of the control animals, pre-treated with either sterile saline or normal chicken erythrocytes and given the same doses of LPS, died.

(iii) The Effect of Administration of Phenylhydrazine hydrochloride (PHND-HCL): It has been shown above that, whereas anaemia per se does/

FIGURE 54. Cumulative Mortality After L.P.S. Injection



does not increase susceptibility of the chicken to LPS, chickens in which the RES may have been previously involved with erythrocyte destruction (erythrophagocytosis) exhibited greatly increased susceptibility to LPS. Therefore, it was decided to investigate the effect of RES erythrophagocytosis, and concomitant anaemia, in combination with LPS.

Preliminary observations indicated that rapid destruction of erythrocytes occurred in the chicken after sub-cutaneous injection of PHND-HCL. The average quantity of erythrocyte lost within 30 hours after injection of PHND-HCL was more than 45 percent of the initial total erythrocyte volume; this was nearly identical with the degree of anaemia observed in chickens infected with S. gallinarum. The various mechanisms of erythrocyte destruction in this chemically-induced anaemia, as postulated by Muirhead et al. (1954), Weed et al. (1961) and Sabine (1964), suggest that, apart from anaemia, erythrophagocytosis and altered reticulo-endothelial activity also occur.

As shown in Table 23, a marked increase in susceptibility to LPS challenge occurred in the chicken 30 hours after sub-cutaneous administration of PHND-HCL. The estimated LD₅₀ was very small (14.5 mg. per Kgm.) as compared with 56 mg. per Kgm. for normal, untreated animals. All the animals injected with 30 mg. of LPS per Kgm. died within 3 to 5 hours after challenge, and 2 out of 5 of such animals died after injection of as little as 10 mg. per Kgm. of LPS. The cumulative mortality pattern of these animals is illustrated in Fig. 54, and their extreme susceptibility to LPS is shown by the marked displacement to the left/

left of cumulative mortality in Fig. 54, which represents the low-dose region.

In contrast, all the control chickens given the same doses of LPS produced hardly any visible signs.

It was also noticed that chickens pre-treated with PHND-HCL were more susceptible to LPS than chickens pre-injected with rabbit erythrocytes (LD_{50} of 14.5 mg., as compared with 21.5 mg.) These animals were far more susceptible than chickens subjected to acute erythrocyte loss alone, (an LD_{50} of 55 mg. per Kgm.). A dose of 30 mg. of LPS per Kgm., which proved fatal for all the PHND-HCL treated chickens, had no demonstrable effect on any of the animals with induced oligocythaemic anaemia.

(iv) The Effect of Injection of in-vivo Sensitized Chicken

Erythrocytes: Following above experiments on susceptibility to LPS after various treatments, the effect of in-vivo sensitized erythrocytes taken from infected chickens was also investigated.

Six hours after i/v injection of in-vivo sensitized chicken erythrocytes, the animals were challenged with different doses of LPS through the intravenous route. Again, as shown in Table 23, all the injected chickens died within 12 hours after challenge with 50 mg. of LPS per Kgm. In contrast, none of the control chickens, which had previously been injected with normal chicken erythrocytes, died.

These animals had a significantly lower LD_{50} of LPS (36 mg. per Kgm.) than animals either rendered anaemic by bleeding before challenge (LD_{50} of 55 mg. per Kgm.) or untreated (56 mg. per Kgm.). Thus, a dose of 30 mg. per Kgm. of LPS, which killed/

killed at least 40 percent of the chickens pre-treated with in-vivo sensitized cells, did not affect either normal chickens or those animals with induced oligocythaemic anaemia. Furthermore, all the animals injected with sensitized chicken erythrocytes were killed with an LPS dose of 50 mg. per Kgm., but none of the untreated, and only one of those rendered anaemic by bleeding, died as a result of subsequent injection of the same dose of LPS. Compared, therefore, with the normal, untreated chickens, chickens pre-treated with in-vivo sensitized chicken erythrocytes showed greatly increased susceptibility to LPS.

The difference and contrasts in susceptibility to LPS in all the above treated animals are illustrated in Fig. 54.

DISCUSSION

The results of these studies have indicated the respective roles which may be played by the processes of anaemia, erythrophagocytis, reticulo-endothelial activity and the liberated endotoxin in the overall pathogenesis and death of chickens infected with S. gallinarum. It is possible that these findings are also applicable to other gram-negative bacterial infections.

Thus, it has been shown that parental administration of antigen-antibody coated homologous (chicken) erythrocytes, heterologous (rabbit) erythrocytes, or subcutaneous injection of phenylhydrazine hydrochloride, markedly increases the susceptibility of chickens to S. gallinarum endotoxin. Although multiple mechanisms may be involved in the altered susceptibility to endotoxin described in these experiments, one factor common to all of the models/

models is the mass presentation of abnormal erythrocytes to the blood stream.

Abnormal erythrocytes are rapidly removed from the circulating blood and then destroyed, apparently by the cells of the reticulo-endothelial system (RES) (Benacerraf, Biozzi, Halpern and Stiffel, 1957; Miescher, 1957; Jandl and Tomlinson, 1958; Cutler, 1961). Nucleated pigeon red cells injected intravenously into heterologous hosts, can actually be seen in reticulo-endothelial cells prior to their disruption (Benacerraf et al., 1957; Halpern, Biozzi, Benacerraf and Stiffel, 1957). Phenylhydrazine hydrochloride interferes with enzymatic activity of erythrocytes (Beutler, 1959) and may also directly damage the erythrocyte membrane (Weed et al., 1961). The affected erythrocytes are more susceptible than normal erythrocytes to removal from the blood stream, presumably by cells of the RES (Jandl and Tomlinson, 1958; Mollison, 1962; Rifkind, 1966).

To explain the altered susceptibility observed in the present experiments, it is suggested that phagocytosis of erythrocytes impairs the metabolic capacity of the RES to detoxify subsequently administered endotoxin. This hypothesis is based on the assumption that the RES is responsible for both the destruction of altered erythrocytes and detoxification or inactivation of the endotoxin. In this connection, it is interesting to note that, apart from the established fact that 'blockade' or damage to the RES increases host susceptibility to endotoxin (Noyes, McInturff and Blahuta, 1959; Formal et al., 1960; Box and Briggs, 1961; Cooper and Stuart, 1961; Suter and Kersanow, 1961; Farrar and Magnani, 1964; Suter, 1964), there is also ample/

ample evidence that a major proportion of endotoxin injected into experimental animals localises within the cells and tissues of the RES, especially in the red-pulp of the spleen and in the Kupffer cells of the liver (Braude et al., 1955; Cremer and Watson, 1957; Carey et al., 1958; Chedid et al., 1963; Golub et al., 1967; 1968). Furthermore, the present results show that it is the group of chickens injected with phenylhydrazine hydrochloride — that is, those with possibly the severest pathological insult because of the occurrence of both anaemia and erythrophagocytosis — that exhibited the greatest increase in susceptibility to endotoxin (Table 23).

The possibility, therefore, exists that ingested erythrocytes interfere with the mechanisms which operate intracellularly to detoxify endotoxin. These conclusions are supported by the observation that in the groups of chickens with probably no occurrence of RES erythrophagocytosis, (for example, those chickens either given homologous erythrocytes or rendered anaemic by bleeding), there was no significant increase in susceptibility to endotoxin as compared with the LD₅₀ for normal, untreated animals. Moreover, phagocytes have been shown to be the main target for endotoxin activity in-vivo (Nelson and Boyden, 1963; Weissmann and Thomas, 1964; Levy and Ruebner, 1967; 1968), as well as in-vitro (Heilman, 1964; 1965, 1968; Kessel and Braun, 1965; Heilman and Bast, 1967; Weiner and Levanon, 1968). More significant, however, in-vivo biological detoxification of endotoxin has actually been shown to be mainly localised in the phagocytic elements of the reticulum cell-rich organs (Wiznitzer et al.,/

et al., 1960; Trapani, Waradvekar, Landy and Shear, 1962; Smith, Rutenburg, Rutenburg and Fine, 1963; Farrar, 1965; Farrar and Corwin, 1966; Rutenburg, Rutenburg, Smith and Fine, 1966; Rutenburg, Skarnes, Palmerio and Fine, 1967) of which the spleen and liver are the primary organs.

Injection of phenylhydrazine hydrochloride accelerates destruction of erythrocytes of many animals (Muirhead et al., 1954; Lowy et al., 1959; Weed et al., 1961; Azen and Shilling, 1963), and was found in the present experiments to produce marked anaemia in the chicken. That the increase in susceptibility to endotoxin in this group of animals cannot be attributed alone to the anaemia produced by this agent is the finding that anaemia per se, such as that caused by acute blood loss, does not increase susceptibility to endotoxin. This suggestion is consistent with the demonstration that there is no increase in susceptibility to E. coli endotoxin in rabbits after reversible haemorrhagic shock (Greisman, 1960). Furthermore, the possibility that the anaemia per se was a lethal factor in this disease was ruled out by the demonstration here that chickens with equivalent, or even more profound degree of anaemia produced by bleeding, did not die. Further evidence that the chicken can tolerate an unusually massive loss of blood is the observation that removal of 85 percent of blood in 2 days does not prove to be fatal (Wirth and Kubasta, 1939).

The demonstration that chickens injected with either heterologous or in-vivo, sensitized erythrocytes show increased susceptibility to the lethal effects of endotoxin confirms the conclusion/

conclusion that impairment of the RES capacity plays an important role in the overall reaction of the animal to endotoxin and probably in the pathogenesis of the disease. The results reveal that susceptibility to the lethal effect of S. gallinarum endotoxin is markedly influenced by the presence of an haemolytic anaemia. This conclusion is supported by the observation that animals in which erythrophagocytosis or 'blockade' of RES have been induced (that is, those injected with thoroughly washed in-vivo sensitized chicken erythrocytes or rabbit erythrocytes) show a greater increase in susceptibility to endotoxin than those in which anaemia alone is present (that is, those with acute blood loss). On the other hand, animals with the concomitant presence of both the 'blockade' of RES and anaemia (that is, those injected with phenylhydrazine hydrochloride) show the most extreme of the susceptibility to endotoxin lethality. It is possible that the reason why chickens injected with phenylhydrazine hydrochloride showed the greatest increase in susceptibility was the actual cellular damage to the reticulo-endothelial cells engaged in endotoxin detoxification, as distinct from the mere functional impairment as a result of erythrophagocytosis and not the added presence of the induced haemolytic anaemia, as suggested above. Although the influence of such a damage, if present, is unknown, it is likely that the enhancement of endotoxin lethality can be entirely explained by the direct toxic action of the drug on the erythrocytes and their subsequent elimination and destruction by the RES. This conclusion/

conclusion is supported by the recent report showing that phenylhydrazine hydrochloride, in adequate amounts, causes specific direct damage to the erythrocyte by oxidation (De Gruchy and Grimes, 1968), apart from the known fact that this drug alters erythrocyte survival by non-immunological means (Muirhead et al., 1954; Beutler, 1959; Weed et al., 1961; Azen and Shilling, 1963). Nevertheless, the possibility still remains that some damage to cells of the RES might have occurred, which conceivably could have affected the response of these animals to subsequent challenge with endotoxin. At present, however, the most reasonable explanation of the altered susceptibility observed in these experiments is that the reticulo-endothelial cells in the livers and spleens of treated chickens are not as effective in inactivating or detoxifying endotoxin as are reticulo-endothelial cells of normal chickens because of reduced efficiency due to blocking and possibly also, anoxic effects resulting from the anaemia.

As with other gram-negative bacterial infections, the pathogenesis of this infection can probably be attributed mainly to the endotoxin liberated by the S. gallinarum organisms in the circulation and tissues. Results from previous chapters have indicated that infection with S. gallinarum directly involved the RES, but the wide variations of the liberated endotoxin in the organs of the RES (Buxton and Davies, 1963) make it quite impossible, on their evidence to relate the lethal outcome and the overall pathogenesis of the disease to any particular pattern of distribution of endotoxin in the tissues. It has also been stressed in previous chapters that probably, the most striking pathologic/

pathologic lesion in this disease is the acute haemolytic anaemia. The present results have enabled the following hypothesis to be formulated to explain the significance of this haemolytic anaemia in the overall pathogenesis of this disease: sensitization of erythrocytes in-vivo leads to their clearance by the RES, with subsequent development of haemolytic anaemia. The continuous, but rapid sequestration of altered erythrocytes eventually results in the functional impairment in the erythrophagocytic and endotoxin-detoxifying functions of the RES of the animal, arising from which an increased susceptibility to endotoxin leads rapidly to death.

Although the effects of anaemia itself (for example, anoxic changes) are not therefore the primary cause of death in this disease, the occurrence of anaemia reflects and indicates the series of complex pathological events which are in progress and which are eventually lethal. All these events are triggered off by the in-vivo sensitization of the erythrocytes and this phenomenon must therefore be regarded as the primary and most significant lesion of this disease.

CHAPTER IXSTUDIES ON SERUM COMPONENTS INHIBITING
ERYTHROCYTE SENSITIZATION BY LIPO-POLYSACCHARIDEINTRODUCTION

The present investigations have shown conclusively that erythrocyte sensitization takes place in-vivo and that this phenomenon is an essential pre-requisite not only in the induction of the haemolytic anaemia associated with acute fowl typhoid, but also in the primary pathogenic mechanism responsible for death.

It has also been suggested that the ability of lipo-polysaccharides (LPS) to interact with human and animal cells and, under appropriate conditions, to bind tightly and irreversibly to their surface receptors, may be a precondition for their toxic action (Neter, 1956; 1963; Springer, 1963; Springer and Horton, 1964; Penhale, 1965). Therefore any way of interfering with this sensitization may have an important effect on the course of this disease.

In view of the number of reports indicating that sensitization of erythrocytes by LPS is inhibited in-vitro by certain factor(s) of human and animal sera (Boyden, 1950; Neter et al., 1953; 1955; 1956; Boyden and Grabar, 1954; De Gregorio and Di Nardo, 1955; Fine, Flocks and Fleichtmier, 1955; Springer and Horton, 1964; Springer and Horton, 1964; Springer et al., 1966; Stewart and McLoughlin, 1968) and possibly also by other tissue fluids as well (Neter et al., 1952), the question therefore arises as to how such an important phenomenon can take place in-vivo.

There/

There have also been no reports so far on, either the conditions necessary for cell sensitization during natural and experimental infection, or on the mode of action of inhibitors in-vivo. These preliminary studies were therefore conducted to:

- (i) determine whether inhibitors were present in chicken serum, and estimate their approximate levels.
- (ii) determine some of their properties and the in-vitro conditions necessary for erythrocyte sensitization.
- (iii) elucidate the probable principal modes of action of the serum inhibitors.
- (iv) determine any changes occurring in serum inhibitor concentrations during the course of acute S.gallinarum infection.

MATERIALS AND METHODS

Sensitizing Antigen LPS: Purified LPS from S.gallinarum was used.

- (i) Preparation of LPS: LPS was prepared and purified by the methods described previously.
- (ii) Standardization of LPS: As one of the main variables in the estimation of serum inhibitor concentration and power, it was essential that the amount of LPS used should be standardized in order to ensure proper comparisons and obtain uniformity in estimation.

Aliquots of normal erythrocyte suspensions were sensitized with serial two-fold dilutions of a known concentration of LPS in an haemagglutination (HA) test, using a standard dilution of S.gallinarum antiserum (vide infra). One 'sensitizing dose' of LPS was taken as the minimum concentration of LPS which sensitizes the erythrocytes for complete haemagglutination. This dose was calculated/

calculated from the total sensitizing doses, which were obtained from the reciprocal of the final HA titre.

S.gallinarum Hyperimmune Serum: Specific hyperimmune serum was prepared in chickens against the O-antigen (LPS) derived from S.gallinarum by a multiple inoculation schedule described previously. After complement inactivation, it was standardized for sensitized erythrocyte detection in the estimation of inhibitor concentration at a dilution of 1:30. The undiluted serum was used in the determination of the inhibitory activity of the chicken serum preparations.

The antiserum was stored without a preservative at -20°C .

Anti-chicken gamma-globulin serum: This was prepared in rabbits by the method described previously.

Haemagglutination (HA) and Antiglobulin HA Tests: The techniques of both tests have been described in previous chapters. The specificity of these tests was checked by means of heterologous group-specific antisera (anti-E.coli O9 and O78 sera).

Inhibitors:

(i) Sources: Sera from normal and infected chickens were the main sources of inhibitor studied.

(ii) Determination of effects of Time and Temperature (Heat) on Serum Inhibitors: Normal chicken serum, at a standard dilution of 1:2 in buffered saline solution, was kept at various temperatures for time periods specified below, in order to determine their heat stability:

- (a) 56°C for one hour.
- (b) 75°C for 30 minutes
- (c) 100°C for 10 minutes.
- (d) /

(d) 100°C for one hour.

The changes, if any, in the serum inhibitor concentrations after these treatments were then determined.

(iii) Standardization of Serum Inhibitor Concentration: In order to achieve uniformity in the determination and comparison of the inhibiting activities after various treatments, standardization of normal serum to obtain a 'minimal inhibiting' dose of the serum components was necessary.

One minimal inhibiting dose (MID) of the inhibitor was taken as the minimum concentration (that is, the highest dilution) of the inhibiting substance which, by preventing the attachment of LPS to erythrocytes, inhibits subsequent haemagglutination by homologous antibody.

The same procedure used above to standardize LPS was utilized here, except that this time, it was the normal serum (1:2 dilution) which was serially diluted and incubated with equal volumes of 10 sensitizing doses of LPS and a suspension of normal chicken erythrocytes. The final inhibiting titre, after the addition of a constant amount of homologous antibody to haemagglutinate the cells, was taken as the total number of inhibiting doses present in the serum dilution used.

Estimation of Inhibitor Concentration (ESI Test): A standard erythrocyte sensitization inhibition (ESI) test was developed for the assay of the concentration of the inhibitor in the serum preparations.

Essentially a haemagglutination-inhibition test, the ESI test involved the use of a standard amount of LPS (which normally contained 10 sensitizing doses); a known quantity or dilution/

dilution of the inhibitor preparation, a saline suspension of normal chicken erythrocytes and a constant dilution of homologous antiserum.

Serial two-fold dilutions of the inhibitor (volume 0.2 ml.) were made in saline, and each dilution was mixed with an equal volume of the standard concentration of LPS. The mixtures were then used as the sensitizing 'antigen', and 0.1 ml. amounts of 1.5 per cent suspension of normal erythrocytes were added to them. The mixtures were incubated at 37°C for one hour, with regular mixing. After being washed 3 times in saline, the erythrocytes were resuspended on 0.2 ml. of saline and then mixed with an equal volume of a dilution of homologous antiserum (1:30) in an HA test.

The inhibitor concentration (ESI titre) was recorded as the reciprocal of the highest dilution of inhibitor preparation that prevented haemagglutination, and hence erythrocyte sensitization.

Control titrations consisted of normal saline in place of inhibitor preparation, or the absence of LPS in the test system.

Antiglobulin-ESI Test: This test consisted of performing an antiglobulin-haemagglutination test (Chapter III) on the washed 'negative erythrocyte buttons' at the end of the ESI test.

Determination of Inhibitory Activity of Serum Preparations (SIA Test):

Apart from estimating the inhibitor concentration of various serum preparations, the inhibitory activity or the inhibiting power of the preparations was also assessed by a method (the 'standard inhibitory activity' (SIA) test) similar to the ESI test, except that the SIA test gives the final inhibitory activity as a haemagglutination titre.

The/

The test consisted of simultaneous addition and incubation at 37°C for one hour, of equal volumes of a fixed quantity of the inhibitor preparation, washed, tightly packed normal erythrocytes and a known dose of LPS. After washing, the erythrocytes were resuspended in saline at 1.5 per cent and then titrated against a serially diluted known amount of homologous antiserum.

The inhibitory activity was recorded as the reciprocal of the highest dilution of antiserum that produced complete haemagglutination.

Investigation of the Action of Inhibitors: To obtain information on the mode of action of the inhibitors, a series of experiments was carried out to determine the effects of the inhibitors on:

- (a) LPS, subsequently used to sensitize erythrocytes.
- (b) Erythrocytes, subsequently sensitized by LPS.
- (c) Erythrocytes, already sensitized by LPS.
- (d) Antibody-combining site of LPS, without preventing its attachment on erythrocytes.

Assay of Serum Inhibitors From Chickens Infected with *S.gallinarum*:

Daily serum samples from acutely infected chickens, and their controls, were assayed for changes in the inhibitor concentrations, using the ESI and antiglobulin-ESI tests.

The sera were from chickens which had been used in previous studies (Chapter III), and subsequently stored at -20°C without preservative.

RESULTS

Preliminary/

Preliminary examination of a variety of randomly selected human and animal sera (for example, horse, bovine, sheep, pig, dog, rabbit, guinea-pig, mouse, human and chicken) showed that a sensitization inhibitor for lipo-polysaccharide (LPS) was uniformly present, but in different concentrations in the individual sera examined. It was also noted that no difference existed between the inhibiting power of ordinary plasma or serum; thus, plasma inhibited erythrocyte sensitization by LPS to the same extent as the serum.

I. Properties of Serum Inhibitors:

(i) Heat stability: Interesting observations were made regarding the heat stability of the serum inhibitors. As shown in Table 24, heating of the serum for one hour at 56°C did not reduce the inhibitory activity, and serum heated at 75°C for 30 minutes was still effective in dilutions of up to 1:128. However, heating the serum for 10 minutes in a boiling water bath (100°C) impaired its inhibitory activity, but only moderately (256 to 64). There was, however, a significant loss of inhibitory activity following boiling for one hour (512 to 8).

Repeat experiments with various serum samples from several breeds of chicken, and other animal species, showed the same pattern and similar effects of heating on the inhibitors.

(ii) Rate of Reaction of the Inhibiting Process: The possibility that the inhibitory effect takes place rapidly was confirmed by the finding that continued contact of the inhibiting serum preparations with LPS for 24 hours at 4°C did not result in substantially greater inhibition than contact for less than 30 minutes at 37°C .

(iii) The/

TABLE 24
EFFECT OF TIME AND TEMPERATURE (HEAT) ON SERUM "INHIBITORS"

TREATMENT OF 10 PER CENT NORMAL SERUM IN SALINE SOLUTION	INHIBITOR CONCENTRATION (ESI TITRE)	
	BEFORE TREATMENT	AFTER TREATMENT
INCUBATION AT 56°C FOR 60 MINUTES	128	128
INCUBATION AT 75°C FOR 30 MINUTES	256	128
INCUBATION AT 100°C FOR 10 MINUTES	256	64
INCUBATION AT 100°C FOR 60 MINUTES	512	8

(iii) The Inhibitor-LPS Combination: On the assumption that the inhibitor combined with the receptor groups of the LPS responsible for attachment to the erythrocytes, it seemed likely that if the inhibitor-LPS combination were not a firm one, then the inhibition titres obtained could vary, within limits of the optima of the reagents, with the concentration of erythrocytes used for sensitization. To test this possibility, the sensitization inhibiting (ESI) titre of a normal serum was determined with 1, 5 and 10 per cent erythrocyte suspensions — the 5 and 10 per cent suspensions being adjusted to 1 per cent for the haemagglutination stage of the test. No difference in titre against LPS was, however, found, confirming the firmness of the inhibitor-LPS combination. This observation does not, of course exclude the possibility that the serum factor caused a permanent chemical alteration of the LPS.

2. Effects of Serum Inhibitors on Subsequent Reaction with Antibodies, as Demonstrated by means of Anti-chicken γ -globulin Serum:

It has been demonstrated previously that the HA test is not sensitive enough to detect minimally sensitized erythrocytes or when sensitized cells are mixed with non-sensitized cells below a certain threshold level (Chapter VI). It is quite likely, therefore, that the standard erythrocyte sensitization inhibition (ESI) and the standard inhibitory activity (SIA) tests, described above for determining inhibitor concentration and power respectively, would not give the true 'end point', since both tests depend on the principle of the HA test.

Experiments were carried out to determine, by use of specific antiglobulin serum, whether erythrocytes treated with inhibitor/

inhibitor-LPS mixtures were capable of subsequent reaction with homologous antibodies.

Chicken erythrocytes were sensitized by S.gallinarum LPS-serum inhibitor mixtures in a standard ESI test. After washing, the erythrocytes were tested for sensitization and globulin adsorption by subjecting them to an antiglobulin-ESI test, using rabbit specific anti-chicken gamma-globulin serum (RACGGS) at a dilution of 1:40.

The experiments revealed that RACGGS agglutinated erythrocytes which had been incubated simultaneously with a mixture of LPS and serum inhibitor, and subsequently had not been agglutinated by homologous antiserum. This finding shows that chicken globulin had been adsorbed by erythrocytes which had been pre-treated with LPS-inhibitor mixture and the absence of previous direct haemagglutination with homologous antibody might have been due to the less sensitive ESI test. Thus, such apparently negative HA-'buttons' of erythrocytes may in fact consist of a population of weakly sensitized cells which was not demonstrable by straight or direct haemagglutination. This interpretation is based on the assumption that specific antibodies would be adsorbed as long as the requisite antigen remains on the erythrocyte surface.

This method of increasing the sensitivity of the test for estimating serum inhibitor concentrations was utilized in subsequent experiments to examine the relationship between inhibitor neutralization and concentration of LPS, and changes which occur during acute S.gallinarum infection.

3. Quantitative Relationship Between the Concentrations of

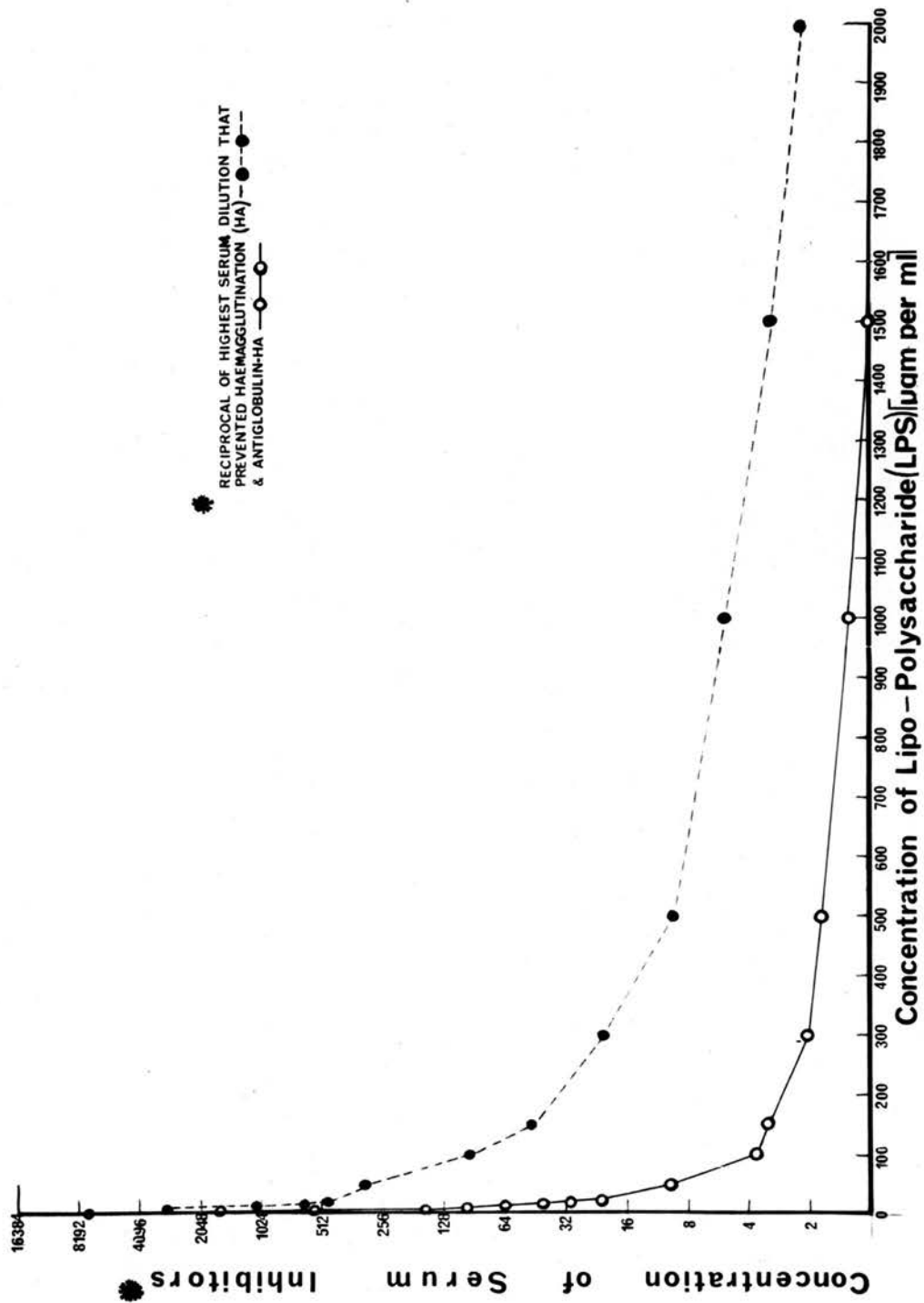
Antigen (LPS) and Serum Inhibitors:

The/

The results of experiments reported so far indicated that the inhibitory effect of serum is dependent on its concentration and may be directly related to the LPS concentration. It was also considered that one of the essential conditions that must be fulfilled if erythrocyte sensitization is to take place in-vivo during the course of bacterial infection is the complete neutralization or inactivation of the inhibitors at the sites of sensitization. Experiments were therefore performed to determine the in-vitro conditions necessary for erythrocyte sensitization, and also the relationship between the inhibitor and LPS concentrations.

The serum inhibitor concentrations in different chicken sera were assayed, using the ESI and the antiglobulin-ESI tests, but the sensitizing doses of the LPS used varied from 1.5 to 2000 μgm per ml. The mean of inhibitor concentrations from 6 different normal serum samples was plotted against decreasing concentrations of LPS, and the results are illustrated in Fig.55. That the antiglobulin-ESI test greatly increases the sensitivity of the ESI test, and hence detection of partial or small amounts of sensitized erythrocytes, was clearly shown. Whereas erythrocyte sensitization of 1500 μgm per ml. of LPS was found (using the antiglobulin-ESI test) to have, in fact, occurred in the presence of serum (1:2 dilution), the use of the ESI test on the same cells, on the other hand, showed complete inhibition of sensitization. Undiluted normal serum was found to inhibit sensitization and subsequent haemagglutination by quite high concentrations of LPS. Indeed, using the ESI test, complete neutralisation of normal serum inhibitory activity was never demonstrated with concentration of LPS even/

FIGURE 55. Quantitative Relationship Between the Concentration of Serum Inhibitors and Sensitizing Antigen (L.P.S.)



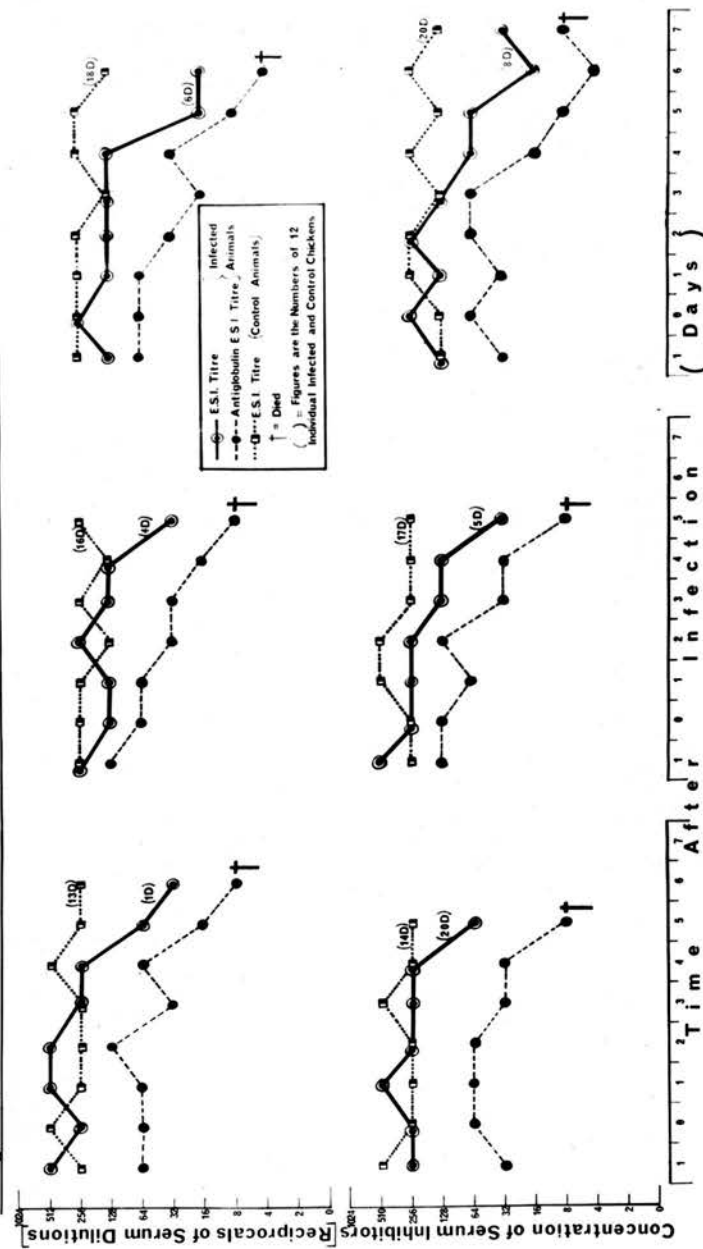
even as high as 2000 μgm per ml. In general, however, increase in serum inhibitor concentrations led to proportional decreases in haemagglutination and increases in haemagglutination-inhibition titres. These semi-quantitative experiments revealed that the more concentrated the LPS, the more serum was required to produce complete inhibition of sensitization of erythrocytes, thus confirming earlier impressions which indicated probable concentration dependence of the inhibitory effect of serum.

4. Serum Inhibitor Changes During Acute *S.gallinarum* Infection:

The results of daily assay of serum inhibitor concentrations of a group of chickens (and their controls) which had been infected with *S.gallinarum* are shown in Fig.56. These representative animals were acutely infected and none survived. Their serum inhibitor concentrations showed slight, but probably significant, decreases towards the time of death. It is seen also that the more sensitive antiglobulin-ESI test demonstrated more profound decreases in inhibitor concentrations during infection than with the ESI test.

It is also of great interest to note that the lowest inhibitor concentrations were detected at a time when the erythrocytes were sensitized and the resulting anaemia was severest (See Figs.10-16 in Chapter III): but it is also significant that inhibitor levels never fell to the point where in-vivo sensitization could conceivably occur in a general and widespread scale throughout the circulation. These results, therefore, indicated that, whilst it is clearly possible to neutralise inhibitor concentrations, this must occur in-vivo in restricted sites and not as a general phenomenon/

FIGURE 56. Changes in Serum Inhibitor Concentrations During the Course of Acute Salm. Gallinarum Infection.



phenomenon in the circulation.

5. The Possible Mode of Action of Inhibitors:

(i) Inhibitory Effects of Serum on Haemagglutination: The present studies have confirmed earlier observations of other workers (vide supra) that serum in some way affected the in-vitro sensitization of erythrocytes by LPS so that subsequent haemagglutination by specific anti-LPS serum did not occur. However, the standard sensitization inhibition test employed per se gave no information on the mode of action of the inhibitors. Therefore, the following experiments were conducted to elucidate the possible mode of action of these inhibitors.

(ii) Influence of Serum Inhibitors of Erythrocyte Coating Effect of LPS: To show whether the inhibitors act directly on LPS by either neutralising or altering those sites on the LPS which combine with the erythrocyte surface to prevent its subsequent adsorption by erythrocytes, LPS (containing approximately 10 sensitizing doses) was mixed with at least 50 MID of serum inhibitors. (For controls, normal saline was substituted for serum inhibitors). After incubation at 37°C for one hour the mixtures (inhibitor-treated LPS) were then used to sensitize normal chicken erythrocytes, which were finally tested for haemagglutination with serially diluted homologous antiserum in a SIA test.

As Table 25A shows, the experiments revealed that the LPS, treated with inhibitors, in contrast to the untreated LPS (saline control), failed to sensitize the erythrocytes as indicated by absent haemagglutination (HA) by homologous antiserum.

These experiments have demonstrated that the LPS after/

TABLE 25

EFFECT OF SERUM 'INHIBITORS' ON THE MECHANISM OF THE PHENOMENA OF ERYTHROCYTE SENSITIZATION AND HAEMAGGLUTINATION

GROUP OF EXPERIMENT	PRIMARY MIXTURE		SUBSEQUENT TREATMENT OF MIXTURE	INHIBITORY ACTIVITY (RECIPROCAL HA TITRE)	
	SERUM 'INHIBITORS'	CONTROL SALINE		SALINE SOLUTION	SERUM 'INHIBITORS'
	PRE-INCUBATED WITH:				
A	<u>SALM. GALLINARUM</u> LPS (SG-LPS)		USED AS 'ANTIGEN' TO SENSITIZE ERYTHROCYTES IN SIA-TEST	1024	<1
B	NORMAL ERYTHROCYTES		WASHED AND SENSITIZED BY SG-LPS; USED IN HAEMAGGLUTINATION (HA) TEST	4096	2048
C	SG-LPS-SENSITIZED ERYTHROCYTES		WASHED AND USED IN HA TEST	4096	4096
D ⁺	<u>SALM. GALLINARUM</u> LPS (SG-LPS)		SERIALY DILUTED AND USED IN HAEMAGGLUTINATION-INHIBITION (HA-I) TEST WITH SG-LPS-SENSITIZED ERYTHROCYTES AND HOMOLOGOUS ANTISERUM	32	32
	-----		SERIALY DILUTED AND USED IN HA-I TEST WITH SG-LPS-SENSITIZED ERYTHROCYTES AND <u>E. COLI</u> ANTISERUM	-	-
	<u>E. COLI</u> LPS (EC-LPS)		SERIALY DILUTED AND USED IN HA-I TEST WITH EC-LPS-SENSITIZED ERYTHROCYTES AND <u>SALM. GALLINARUM</u> ANTISERUM	-	-
E	<u>SALM. GALLINARUM</u> ANTIBODY		SERIALY DILUTED AND USED WITH SG-LPS-SENSITIZED ERYTHROCYTES IN HA TEST	2048	2048
			ABSORBED WITH SG-LPS-SENSITIZED ERYTHROCYTES AND 'SUPERNATANT' TITRATED FOR RESIDUAL ANTIBODY		2
+ = TITRES DETERMINED BY ANTIBODY-NEUTRALISATION IN HAEMAGGLUTINATION-INHIBITION TEST					

after previous contact with the inhibitors, failed to sensitize erythrocytes for agglutination with homologous antibodies as evidenced by complete inhibition of the HA. These inhibitors may, therefore, act either by neutralising or by altering the erythrocyte-sensitizing sites on the LPS.

(iii) Effects of Inhibitors on Erythrocytes and Subsequent Sensitization by LPS: The possibility existed that these inhibitors may initially act on the erythrocytes, rather than on the LPS, to inhibit HA in 2 possible ways:

(a) their interaction with the LPS-combining sites on the erythrocytes, therefore interfering with subsequent adsorption of LPS by the cells.

(b) Rendering the erythrocytes inagglutinable, without actually preventing LPS adsorption.

The former principle was investigated by performing a series of experiments to determine the effects of inhibitors on the erythrocytes which were subsequently sensitized by LPS.

0.15 tightly packed erythrocytes was incubated for 30 minutes at 37°C with approximately 250 MID of serum inhibitors. (Control experiments were performed with normal saline replacing the inhibitors). The cells were then thoroughly washed and subsequently exposed to LPS to be sensitized in the usual manner. Aliquots of erythrocyte suspension in saline were then mixed with serially diluted homologous antiserum and HA determined.

The experiments revealed that the inhibitors did not substantially affect haemagglutination when they were allowed to act initially on the erythrocytes prior to sensitization with LPS. (Table 25B). These results therefore showed that the inhibitors do not/

alter the surface of erythrocytes, thereby interfering with their subsequent adsorption of LPS.

(iv) Effects of Inhibitors on Erythrocytes Already Sensitized:

As previously mentioned, the other possible effect of the action of inhibitors on erythrocytes is to render them inagglutinable without actually preventing attachment of LPS to the erythrocyte surface. This possibility was examined by experiments designed to show the effect of inhibitors on erythrocytes already sensitized.

Erythrocytes, sensitized with LPS, were incubated with the inhibitors in the manner described above. After washing they were mixed with dilutions of homologous antiserum to agglutinate. The results, shown in Table 25C again confirmed the complete lack of inhibitory action on erythrocytes as indicated by the absence of any inhibition of haemagglutination. The inhibitors, therefore, do not render the erythrocytes inagglutinable by homologous antiserum.

(v) Effects of Inhibitors on Antibody-neutralising Capacity of LPS: It is conceivable that the inhibitors may interact with LPS, and by altering it, shield its antibody-combining site without preventing its attachment to the erythrocyte surface. This possibility was studied mainly in haemagglutination-inhibition (HA-I) assays by determining the effect of inhibitors on the antibody-neutralising capacity of LPS.

10 sensitizing doses of LPS were exposed to 50 MID of serum inhibitors, and after incubation for one hour at 37°C, the mixture was serially diluted in saline (volume 0.1 ml.). Then, 0.2 ml. volumes of a known amount of homologous antiserum were added/

added to the LPS -inhibitor mixture and then incubated for a further 30 minutes. Finally, 0.2 ml. amounts of erythrocytes, sensitized in-vitro with 10 sensitizing doses of LPS, were added to the mixtures and incubated again.

Control experiments consisted of untreated LPS in saline (that is, without the inhibitors). Specificity of these reactions was checked by incorporating a heterologous system in the form of E.coli LPS and its homologous antiserum.

The results of these experiments are shown in Table 25D, and they reveal that no decreases in the haemagglutination-inhibition (HA-1) titres, as compared with the control experiments, occur after exposing the LPS to inhibitors. These observations indicate that there is no interference with the antibody-neutralising capacity of the LPS by the inhibitors. The results also show that this antibody-neutralisation is specific, since the heterologous system, involving E.coli LPS and its antiserum, was incapable of showing any HA with S.gallinarum LPS and its antiserum.

The above results strongly indicate that the inhibitory action on haemagglutination is neither due to the shielding of the antibody-combining sites of the LPS, nor to the alteration of LPS in such a way as to preclude its reactivity with the homologous antibody. These observations therefore show that the inhibitors do not destroy or have any effect on the antigenicity of the LPS.

DISCUSSION

The present study confirms previous observations that a non-specific inhibitor for endotoxin, used as an erythrocyte sensitizing/

sensitizing antigen, commonly occurs in normal human and animal sera (Neter et al., 1952; 1953; 1955; 1956; De Gregorio and Di Nardo, 1955; Springer and Horton, 1964; Springer et al., 1966; Stewart and McLoughlin, 1968). The present investigations have, however, shown that the LPS attachment of erythrocytes was inhibited by heat-stable components of the serum, which, under in-vitro physiological conditions, probably interact with, or alter the LPS in such a way as to prevent coating of erythrocytes. The extreme heat stability of these serum components was indicated by the finding that normal chicken serum, heated at 100°C for 30 minutes, was still effective as inhibitor in dilutions up to 1:80.

The nature of the inhibitor(s) was not definitely determined, but from studies not recorded here, its Sephadex G200 fractionation and sodium sulphate precipitation characteristics indicated that the inhibitors are non-antibody serum components. This conclusion was based on the finding that significant inhibitory activity could not be demonstrated in the albumin and gamma-globulin fractions of the serum.

The actual inhibiting process was found to be relatively rapid, and its interaction with the LPS was also determined to be reasonably firm. Furthermore, it was found that the serum inhibitory effect was dependent on the concentration of LPS: the more concentrated the serum, the more endotoxin is required to effect complete haemagglutination.

The present experiments have demonstrated that the serum inhibitors do not interfere with the haemagglutination process by the antibody, but the standard sensitization test per se gives no information on the mode of action of the inhibitors. An inhibitor/

inhibitor may exhibit a number of principal effects. It may (a) neutralise (block) or alter (chemically) those sites on LPS which combine with the erythrocyte surface; (b) block or alter the LPS-receptor sites on the erythrocyte surface; (c) interact with erythrocyte in such a way as to render it inagglutinable, without actually preventing attachment of LPS to the erythrocyte surface; or (d) interact with the LPS by shielding its antibody-combining sites without preventing its attachment to the erythrocyte surface. Finally, an inhibitor may exhibit a combination of these properties. Experiments designed to elucidate the principal modes of action of the serum inhibitors, and depicted in Table 25, strongly indicate that these inhibitors primarily act by preventing the attachment of LPS to erythrocytes, and this could be achieved by either the blocking (neutralising) or chemically altering the receptor sites on the LPS. Inhibitors were found not to interact with the erythrocytes, and probably also, they may have no other interfering effect.

The conclusion that the inhibitors act by altering the LPS in such a way as to block or alter its erythrocyte-sensitizing sites is supported by the finding that the inhibitors, after previous contact with LPS, completely inhibited haemagglutination (Table 25A). In contrast, they did not affect haemagglutination when they were first allowed to act either on normal erythrocytes (Table 25B) or on erythrocytes already sensitized (Table 25C). These observations indicated clearly that the inhibitors do not attach to (block) or alter the surface of erythrocytes, thereby interfering with its subsequent adsorption of LPS. This conclusion is in agreement with the observations that the inhibitors did not render/

render the sensitized erythrocytes inagglutinable by homologous antiserum, and had also not prevented subsequent LPS adsorption by erythrocytes when they were allowed first to act on normal erythrocytes prior to sensitization with LPS.

The failure of the inhibitors to alter the antibody-neutralising capacity of the LPS, as evidenced by the absence of any changes in the haemagglutination-inhibition titres (Table 25D) would appear to indicate that the inhibitory action is neither due to the shielding of the antibody-combining sites of the LPS, nor to an alteration of the LPS in such a way as to preclude its reactivity with homologous antibody. The results also demonstrate that the inhibitors do not destroy or have any effect on the antigenicity of the LPS; they suggest that the inhibitors combine with, or alter, those groups on the sensitizing bacterial antigen, other than those responsible for combination with specific antibody. These groups have been indicated by the present experiments to be those directly responsible for combination of LPS with the erythrocyte. In this connection, the possibility must be considered that the LPS-combining groups on the inhibitors are, in fact, identical with the LPS-combining groups on the erythrocyte. As yet, little is known about the nature of the erythrocyte receptors to which LPS attach, or which parts of the LPS molecule fixes itself to the erythrocyte surface.

The first efforts to identify an erythrocyte receptor responsible/

responsible for adsorption of bacterial antigens (Mycobacterial polysaccharides) appear to be those of Boyden (1950), who noted that an alcohol-ether (3:1) extract of horse erythrocytes contained some substance(s) capable of inhibiting competitively, the sensitization of horse erythrocytes by mallein to agglutination by anti-mallein sera. Recent studies by Springer and colleagues (Springer et al., 1966; Pavlovskis and Springer, 1967), have, however, established that pure "LPS-receptor" from human erythrocytes could be obtained by extracting the homogenised stroma with n-butanol: water (1:1), pH 8 at 37°C. Activity was found to reside in the aqueous phase. An imaginative investigation, which has yielded at least a limited amount of information on the mode of attachment of bacterial antigens (E.coli LPS) to erythrocytes is that of Davies and colleagues (Davies et al., 1958), who based their studies on the well-known observations that both heating and alkali treatment of bacterial polysaccharides enhance the sensitizing activity (Neter, 1956). The absence of a direct relation between coating ability and the lipid component of LPS was noted, and the effect of alkali was shown to be the removal of O-acetyl. They hypothesised that the O-acetyl residues inhibit adsorption on to erythrocytes, and considered it unlikely that bacterial lipid plays a significant part in prevention of erythrocyte sensitization. Nevertheless, Tsumita and Ohashi (1964), with reference to tubercular polysaccharide, have since presented convincing evidence in support of the contention that the lipid moiety of LPS is essential for the sensitization of erythrocytes, and that cholesterol in erythrocyte stroma seems to have a significance in the process of sensitization. Furthermore/

Furthermore, the recent finding that the receptors responsible for the O- and Vi-antigen fixation are the lipoids of the erythrocyte surface (Karalnik, 1965; 1967) lends further support to this possibility. It is also worthy of note that serum albumin, α_2 , β -lipoproteins, cholesterol, lecithin, and other lipid-rich substances, have been found to be potent inhibitors (Neter et al., 1952; 1953; 1955; De Gregorio and Di Nardo, 1955; Springer and Horton, 1964; Springer et al., 1966).

In spite of the inhibitory effect of serum, in-vivo sensitization of erythrocytes has been observed, both as a common occurring phenomenon during acute S.gallinarum infection of chickens and less commonly, in other gram negative bacterial infections of man and animals. The problem of erythrocytes being sensitized in-vivo in the presence of these serum inhibitors has only slightly been elucidated by the following observations: that significant reductions in the serum inhibitor concentrations occur during acute fowl typhoid; significantly, these decreases coincide with the time of detection of sensitization and severe anaemia (Fig.56). It is also of interest to speculate if these decreases are related to the marked reductions in serum cholesterol and phospholipid levels in chickens infected with S.pullorum (Lambe and Holtman, 1960).

There have been no previous reports on the conditions necessary for in-vivo erythrocyte sensitization during natural or experimental infection, but the present observations strongly suggest that at least 2 necessary conditions must be fulfilled in order to permit the coating effect by bacterial lipo-polysaccharides to occur/

occur in-vivo:

(a) large amounts of the polysaccharide must be present in the vicinity of the cells to be sensitized;

(b) serum inhibitors must be below a certain critical level, or must be neutralised by LPS in these restricted areas.

It is significant that inhibitor levels during acute fowl typhoid never fell to the point where in-vivo sensitization could conceivably occur in a general and widespread scale throughout the circulation. These observations, therefore, indicated that, whilst it is clearly possible to neutralise inhibitor concentrations, this must occur in-vivo in restricted sites and not as a general phenomenon in the circulation. This suggestion is favoured by the finding that even with large quantities of LPS, complete neutralisation or inactivation of inhibitory activity present in the undiluted normal chicken serum was never demonstrated by using the ESI test alone and (Fig.55), unless the more sensitive anti-globulin-ESI test was also employed; quantities of endotoxin, larger than those indicated in the present results, tended to lyse the normal erythrocytes. The fulfilment of the above conditions for in-vivo sensitization could also explain the paradox of co-existence of in-vivo sensitized erythrocytes and significant levels of inhibitors in the circulation of chickens with acute fowl typhoid.

Since serum inhibitors probably effectively prevent access of endotoxins to cells remote from the actual site of bacterial multiplication, it would seem likely that they could well be playing an important role in the defence of the body against some of the results of gram-negative bacterial infection. This assumption/

assumption is in agreement with the suggestion that the ability of LPS to interact or attach itself to human and animal cell surfaces may be a pre-condition for ~~their~~^{its} toxic action (Neter, 1956; Springer, 1963; Springer and Horton, 1964; Penhale, 1965). It is possible that, in general, the main biologic significance of the inhibitors is physiological, and the blocking or altering action may be contributing to the inactivation of a wide spectrum of toxic bacterial substances.

CHAPTER XGENERAL DISCUSSION AND CONCLUSIONS(A) GENERAL DISCUSSION:

The studies reported in this thesis confirm previous reports that in-vivo sensitization of erythrocytes occurs concurrently with a severe anaemia during acute fowl typhoid (Buxton, 1959 a,b; 1960), and they establish conclusively that the main in-vivo sensitizing factor is a specific bacterial lipo-poly-saccharide which is directly responsible for the initiation and induction of the anaemia. This anaemia is also clearly shown here to be haemolytic, and extravascular. Although previous reports by Buxton (1959 a; 1960) have suggested that increased destruction of erythrocytes may be responsible for this anaemia, the present studies have presented direct evidence to support this suggestion.

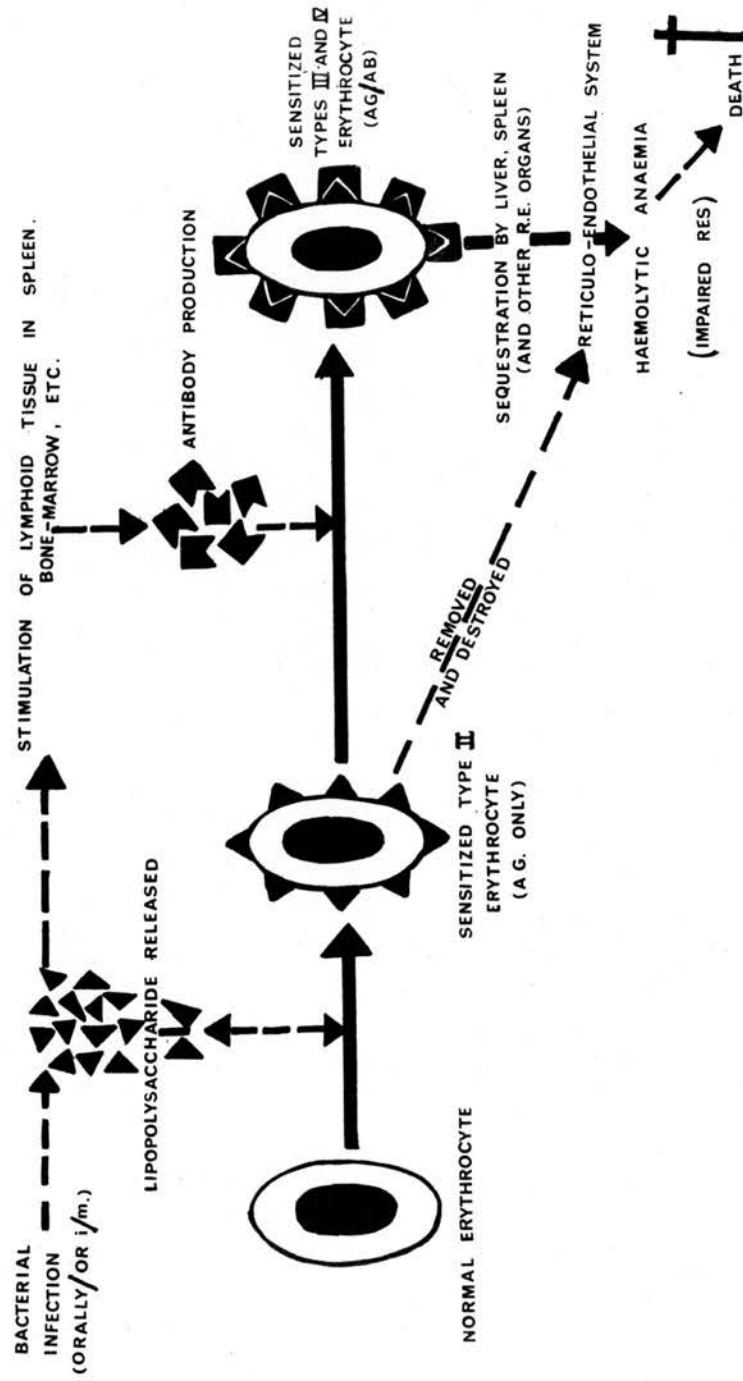
The probable mechanism of this anaemia has not been determined until the present studies, which suggest that immunological factors may be directly involved in the destruction of the erythrocytes. Unlike the conclusions to the contrary by Ho and Kass (1958) which were drawn for experiments in rabbits, immunological mechanisms may be responsible for the haemolytic anaemia observed in chickens after single or multiple intravenous administration of S.gallinarum endotoxin. The possibility that endotoxins owe their haemolysis-inducing property not only to their antigenicity and ability to sensitize erythrocytes in-vivo, but also the host's relative insusceptibility to endotoxin, offers an elegant explanation for this striking induction of an immuno-haemolytic anaemia by endotoxin/

endotoxin injection in the normal chicken and also the presence of the dual-phenomena of in-vivo sensitization and haemolytic anaemia in S.gallinarum infected chickens — phenomena which, it appears from published evidence, are not so readily demonstrable in other species.

It has been shown in the present studies, that relative susceptibility of chickens to endotoxins occurred only after the animals have been pre-injected (and therefore sequestered) with heterologous or in-vivo sensitized erythrocytes. The animals, however, became highly susceptible if both phenomena of erythrophagocytosis (and hence, presumably, RES blockaded) and anaemia were present. In contrast, the anaemia per se did not increase the susceptibility of the animal to endotoxin. These observations lead to the important conclusion that as a result of in-vivo sensitization the susceptibility of chicken to endotoxin is greatly increased, and this is therefore an extremely significant factor in the overall pathogenesis of this disease. These important findings explain the anomaly why a species, which is so refractory to endotoxin, can be infected with, and is extremely susceptible to, a gram-negative endotoxin-producing organism. Fowl typhoid infection is therefore no exception to the generally accepted hypothesis that the endotoxic component is the chief factor in the pathogenesis of these gram-negative bacterial infections.

Taken together, the findings reported in this thesis have enabled a plausible hypothesis to be formulated to account for the increased destruction of erythrocytes during acute fowl typhoid; the postulated mechanism of the haemolytic anaemia is illustrated in Fig.57. Evidence bearing on the mechanism can be adduced by hypothesising/

FIGURE 57. POSTULATED MECHANISM of the HAEMOLYTIC ANAEMIA



hypothesising rapid phagocytosis of erythrocytes which have become coated (sensitized) with specific bacterial lipo-polysaccharide and/or specific bacterial polysaccharide-antibody complex. The specific bacterial polysaccharide released during infection sensitizes both the erythrocytes in-vivo and induces de novo specific antibody; the interplay between these processes causes rapid sequestration and destruction of erythrocytes, mainly by the RES. The suggested mechanism resembled the Type II hypersensitive reaction of Gell and Coombs (1968), and complement may or may not be involved. A similar mechanism has been shown to be responsible for the penicillin (Hapten)-induced immuno-haemolytic anaemia (Levine and Redmond, 1967; Dacie, 1968; Croft, Swisher, Gilliland, Bakemeier, Leddy and Weed, 1968; Carstairs, 1968; Worlledge, 1969).

Although the evidence presented here clearly suggests that the above mechanism is the most probable one, there are also a number of possible ways by which, in general, a gram-negative bacterial infection could damage erythrocytes in-vivo, and thus lead to their rapid destruction and consequent production of a haemolytic episode:

(1) By the direct effect of bacteria on erythrocytes during infection. It is possible that a bacterial product may bring about direct agglutination of the erythrocytes, as a wide variety of the enteric bacteria (for example, Salmonella, E.coli, Brucella and Vibrio) have been reported to be capable of acting in this way in-vitro (Neter, 1956). Whether this mechanism often operates and brings about haemolysis in-vivo during infection is unknown. It is unlikely, however, that this mechanism occurs in-vivo to any great extent in general/

general, and if it does, it is doubtful if it is very important as a cause of increased erythrocyte destruction in acute enteric infections. Certainly this phenomenon was not observed in this disease.

(2) By T-antigen transformation (the Thomsen-Friendenreich and polyagglutinability phenomena). The phenomenon in which the erythrocytes undergo transformation by bacterial enzymes so as to become polyagglutinable by normal serum, irrespective of the blood groups, can occur both in-vitro and in-vivo. The in-vitro transformation, known as the Thomsen-Friendenreich (T-F) phenomenon, is normally seen in old blood samples with bacterial contamination (Reepmaker, 1952; Neter, 1956; Springer, 1963). The consensus of opinion seems to be that the very similar, but more uncommon, in-vivo phenomenon (termed polyagglutinability) and the T-F phenomenon are identical because of the outstanding fact that in most cases of polyagglutinability a bacterial infection can be shown to be present (Mishra, 1959; Chorpenning and Hayes, 1959; Evens, Bingham, Hickey and Hassett, 1959; Evans, Bingham and Weiser, 1963). It is very likely that enzymes are liberated by the infecting organisms which activate the T-agglutininogen and transform the erythrocytes in-vivo (Ejby-Poulsen, 1954). Whether this phenomenon of polyagglutinability can subsequently cause destruction of erythrocytes has not been proved, and it remains a possible rare cause of haemolysis, at least in human bacterial infections (Dausett, Moullec and Bernard, 1959). Indeed, preliminary experiments conducted during the present studies showed that polyagglutinability did not occur during fowl typhoid.

(3) By splenic hypersequestration due to splenomegaly, as a direct consequence of generalised hypertrophy of the RES. The possibility that the hypertrophy of the RES (especially the spleen) which occurs as/

as a consequence of bacterial infections, particularly in relation to human typhoid and others (Jandl et al., 1957; Jandl, Jacob and Daland, 1961; Craddock, 1962; Wagner et al., 1963) may lead to increased destruction of erythrocytes has often been speculated upon. However, it has proved very difficult to obtain satisfactory evidence in its favour, although more recent clinical and experimental work has provided further evidence which support the above concept (Sherman and Friedell, 1962; Wagner et al., 1963; Jacob, 1966).

(4) The auto-immune alteration of erythrocytes by the infecting bacteria. Genuine anti-erythrocyte antibody haemolytic anaemia has often developed following virus infections (Dacie, 1963), but it still remains uncertain whether bacterial infection, by itself, is capable of initiating an auto-immune haemolytic anaemia. However, this appeared to be possible in a few cases in Dacie's patients (Dacie, 1963; see his Table 14). Nevertheless, the case for this mechanism is not very convincing, and certainly is not proven because the presence of positive direct antiglobulin tests cannot, on their own, be accepted as evidence that anti-erythrocyte antibodies are being produced; a reaction between immune antibody and adsorbed bacterial antigen (polysaccharide) has to be excluded, as has been shown in the present studies.

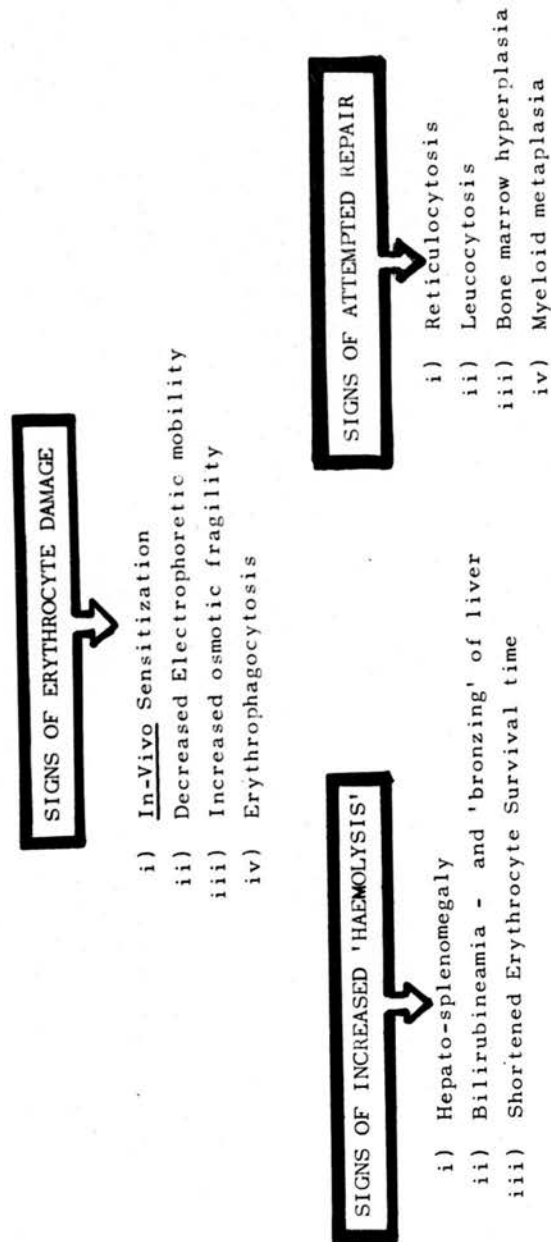
The observed facts which support the hypothesis offered above for the induction of the anaemia of acute fowl typhoid include:

- (a) The failure to demonstrate anti-erythrocyte antibodies.
- (b) The presence of specific bacterial polysaccharide, both in the serum and on erythrocyte surface.
- (c) The presence of antibodies to these polysaccharide antigens.
- (d)/

(d) The development of anaemia following injection of either polysaccharide alone or together with anti-polysaccharide antibodies into normal animals. More important, however, direct evidence of increased erythrocyte destruction (for example, signs of erythrocyte damage, increased haemolysis and attempted repair) were all demonstrated during acute fowl typhoid. A scheme, summarising the observed and postulated consequences of immune destruction of erythrocytes in this disease, is shown in Fig. 58. Every sign indicated in this scheme was either observed or determined during investigations into the various aspects of this disease. The results provide convincing evidence in support of the suggestion that increased destruction of erythrocytes alone may account for the severity of the observed haemolytic anaemia.

From the experiments on in-vivo destruction of erythrocytes damaged by immune mechanisms, it is clear that the fate of such cells is subject to considerable variation as to the rate and sites of destruction. From the present studies with ^{51}Cr labelled-erythrocytes, and other evidence by Jandl et al. (1957) Mollison and Hughes-Jones, (1958) Mollison, (1959; 1962; 1967) Jandl and Kaplan (1960) and Mollison, Crome, Hughes-Jones and Rochna (1965), the following general principles can be established: as a rule, erythrocytes which are severely damaged, or are removed very rapidly from the circulation, are sequestered primarily in the liver. This hepatic sequestration is probably by virtue of the blood flow pattern of the liver. The spleen appears to be more concerned with less drastically injured erythrocytes, and removal of cells proceeds more slowly. This unique capacity of the spleen to recognise, sequester and destroy erythrocytes so altered, appears to/

FIGURE 58. CONSEQUENCES OF ERYTHROCYTE DESTRUCTION *



* The signs are not specific for this disease, and may be seen in a variety of haemolytic anaemias

to depend principally upon the distinctive anatomic arrangement of the splenic reticulo-endothelial cells and vasculature (Barcroft and Poole, 1957; Harris, McAllister and Pranker, 1958; Weiss, 1962; Rifkind, 1965; Jandl, Files, Barnett and MacDonald, 1965).

Commonly it is the fixed macrophages of the RES that are responsible for the final stages of the extravascular haemolysis and erythrophagocytosis. The final stages of in-vivo destruction of erythrocytes, following the initial trapping of the injured erythrocytes, are still poorly understood, but recent reviews by Leddy (1966) Rifkind (1966) and Jandl (1966) summarise and evaluate the present knowledge and clarify the general and selective in-vivo mechanisms implicated in the final stages of the destruction of erythrocytes.

There have been no previous reports on the conditions necessary for in-vivo erythrocyte sensitization during natural or experimental infection, but the present findings, particularly serum inhibitor changes, strongly suggest that the necessary conditions which must be fulfilled in order to permit in-vivo erythrocyte adsorption of specific bacterial polysaccharide to occur must include:

- (a) the presence of large amounts of polysaccharide in the vicinity of the cells to be sensitized;
- (b) the neutralisation of inhibitor by LPS in these areas, or the reduction of serum inhibitors below a certain critical level in these restricted sites. It is impossible to decide how these conditions are fulfilled during acute fowl typhoid, but from the available evidence, it is reasonable to suggest that in-vivo sensitization occurs in areas of maximum infection; for example, in/

in the RES and intestinal walls. In these sites of massive bacterial multiplication, erythrocytes may be exposed to relatively high concentrations of polysaccharide during the course of circulation through them.

Serum inhibitors of sensitization may probably be playing an important role in the defence of the body against some of the results of gram-negative bacterial infection by effectively preventing access of endotoxins to cells; the blocking or altering action of the inhibitors may therefore be contributing significantly, but indirectly, to the inactivation or detoxification of the endotoxin. The demonstration of this inhibitory effect of serum suggests that concentrated preparations of these serum components (non-gamma-globulins) may be of therapeutic value, as a general prophylactic measure, in gram-negative bacterial infections.

Reports by other workers have suggested that the phenomenon of in-vivo erythrocyte sensitization may have other roles to play in the host response to endotoxin. For instance, Gramlich and Muller (1963), Springer and Horton (1964), and Springer (1967) have argued that this sensitization of erythrocytes may point to a transport or detoxifying function of the individual erythrocyte pool, whereas the findings of Hill and Weiss (1964) are in favour of a causal relationship between erythrocyte sensitization and the lethal effect of endotoxin. Hill and Weiss found a relationship between the high degree of susceptibility of A-strain mice to the lethal effect of endotoxin and the affinity of these animals' erythrocytes for killed salmonellae and their lipopolysaccharide components. The recent finding of high susceptibility of strain A mice to both endotoxin/

endotoxin and sub-lethal endotoxin-erythrocyte mixtures (Heppner and Weiss, 1965) confirms the previous observations of Hill and Weiss (1964).

The suggestion that endotoxins owe their lethal activity, not only to their antigenicity, but also their ability to sensitize or to attach to tissue cells (Neter, 1956; 1963; Springer, 1963; Springer and Horton, 1964; Penhale, 1965) offers an elegant explanation for the concept that allergic reactions may play a significant part in the pathogenesis of gram-negative bacterial infections. Buxton and Allan (1963) and Buxton and Davies (1962) have previously suggested that hypersensitive reactions are involved in the pathogenesis of S.gallinarum infection in chickens. Certainly, the striking similarity between the clinical signs of acute fowl typhoid (Chapter II) and anaphylactic shock in chickens (Makinodan et al., 1952; Aronson et al., 1961) indicates that, rather than resulting from toxæmia, the main manifestations of the disease can be explained to a large extent by a hypersensitive reaction. Evidence has also been presented by Buxton and colleagues that the white blood cells could be shown to be passively sensitized with antibody, rendering them susceptible to a hypersensitive (anaphylactic) reaction (that is, a Type I according to Gell and Coombs' (1968) classification). The present observations, however, demonstrating, as they do, the ease of adsorption of bacterial lipopolysaccharide on to erythrocytes and possibly other tissue cells (leucocytes) as well (see reviews by Zweifach and Janoff, 1966; and Nowotny, 1969), would suggest that the symptomatology and the pathogenesis of S.gallinarum infection in chickens may be the direct result of a hypersensitive reaction in which primary attachment of bacterial antigen to the cell surface sensitises/

sensitises it to the cytotoxic effect of homologous antibody (Type II Gell and Coombs' hypersensitivity). Similar allergic factors may also be operating in other gram-negative bacterial infections.

The highly significant findings which have evolved from studies on this disease point to several intriguing lines of investigation, and in the light of the current emphasis on all aspects of comparative medicine and the use of bio-medical models, it is concluded that the chicken and the fowl typhoid syndrome have useful roles to play, as they offer ^{an} useful animal model for the study of both the pathogenesis of gram-negative bacterial infections and the mechanisms which ultimately lead to haemolytic episodes in these infections.

(B) CONCLUSIONS:

The conclusions reached as a result of the present studies are that:

- (1) Adult chickens are extremely susceptible to infection with S.gallinarum, and one of the most conspicuous pathological changes resulting is the development of a very severe, macrocytic, normo-chromic, haemolytic anaemia.
- (2) The erythrocytes regularly become sensitized during the course of infection, and these cells have greatly increased osmotic fragility.
- (3) The in-vivo sensitizing factors are specific bacterial polysaccharide (LPS) and homologous antibody.
- (4) This phenomenon is intimately associated with a critical stage of the development of the anaemia.
- (5) The erythrocytes are 'maximally' or heavily sensitized in-vivo and the cells recovered from infected animals consist of a mixed heterogeneous/

heterogeneous population of a small minority of 'maximally' sensitized erythrocytes and large numbers of non-sensitized immature erythrocytes (reticulocytes).

(6) Distinct types of in-vivo sensitization occur, and a relationship exists between the type of in-vivo sensitization, the severity of the haemolytic episode and the duration of infection.

(7) Antibody-mediated destruction of erythrocytes probably accounts wholly for the severity of the anaemia observed.

(8) The erythrocyte life-span of an infected chicken is reduced to approximately one-sixth that of the normal animal, and the spleen and liver are the main organs responsible for the final destruction of these cells.

(9) The site of reticulo-endothelial destruction is dependent upon the degree of erythrocyte surface alteration by bacterial polysaccharide. Erythrocytes which are maximally sensitized in-vitro are rapidly cleared in normal chickens; clearance by the liver is greatly enhanced by the presence of homologous anti-endotoxin antibodies. Minimally in-vitro sensitized cells are, however, destroyed relatively slowly by the spleen, and the presence of homologous antibody does not seem to affect the rate of clearance.

(10) Haemolytic anaemia can be produced experimentally in chickens by single or repeated injections of endotoxin alone after a latent period of 4 - 5 days.

(11) Haemolytic anaemia can be induced within a few hours of the injection of endotoxin in combination with homologous antibody.

(12) Anaemia, as it occurs in fowl typhoid, is an extremely significant factor in the overall pathogenesis of the disease because it results/

results in RES blockade. Anaemia per se does not increase susceptibility of chickens to endotoxin, but as a result of in-vivo sensitization and phagocytosis of erythrocytes by the RES, the susceptibility of these animals to endotoxin is greatly increased.

(13) The above findings may explain the anomaly that a species which is so refractory to endotoxin can be infected with, and is extremely susceptible to, an endotoxic-producing organism (S.gallinarum).

(14) In addition to erythrocytes, sensitization may occur elsewhere in other tissues and may play a significant part in the overall pathogenesis of this disease.

(15) Normal chicken serum contains high levels of heat-stable, non-specific, non-immunoglobulin substances which inhibit erythrocyte sensitization by bacterial lipo-polysaccharide. These serum inhibitors act by interacting with the lipo-polysaccharide in such a way as to prevent coating of erythrocytes, either by neutralising or altering those sites on lipo-polysaccharide which combine with the erythrocyte surface.

(16) In-vivo sensitization does not occur in a general and wide-spread scale throughout the circulation, but in restricted areas where large amounts of bacterial lipo-polysaccharide must be present, thus neutralising the inhibitors.

(17) Fowl typhoid syndrome is an ideal experimental model for the study of the mechanism of anaemia of acute microbial infections of both human and animal origin, especially those caused by gram-negative bacteria.

REFERENCES

- Agarwal, K.N. and Hjertén, S. (1964), *Acta endocr.*, Copenh. 47,
(Suppl. 93) 53.
- Alexander, W.R., (1958), *Immunology*, 1, 217.
- Allan, D., Duffus, W.P.H. and Higgins, D.A., (1968), *Immunology*, 14,
575.
- Allan, R.L., (1969), Ph. D. Thesis, Univ. of Edinburgh.
- Angers, J. and Rottino, A., (1961), *Blood*, 17, 119.
- Archer, R.K. (1965), 'Haematological Techniques for use on Animals',
Blackwell Sci. Pub., Oxford.
- Aronson, F.R., Bilstad, N.M. and Wolfe, H.R. (1961), *Poult. Sci.* 40,
319.
- Atkins, E., (1960), *Physiol. Rev.* 40, 580.
- Atkins, E. and Snell, E.S. (1964), In 'Bacterial Endotoxins', eds.
Landy, M. and Braun, W., Rutgers Univ. Press, New Brunswick,
p. 134.
- Azen, E.A. and Shilling, R.F. (1963), *J. Lab. clin. Med.* 62, 59.
- Azen, E. A. and Shilling, R.F. (1964), *J. Lab. clin. Med.* 63, 122.
- Bakemeier, R.F. (1965), *J. Immun.* 95, 880.
- Bangham, A.D., Flemans, R., Heard, D.H. and Seaman, G.F. (1958),
Nature, Lond. 182, 642.
- Barcroft, J. and Poole, L.T. (1957), *J. Physiol., Lond.* 64, 23.
- Bateman, J.B. and Zellner, A. (1956), *Archs. Biochem.* 60, 44.
- Bateman, J.B., Zellner, A., Davis, M.S. and McCaffrey, P.A. (1956),
Archs. Biochem. 60, 384.
- Beaudette/

- Beaudette, F.R. (1930), Proc. XIth Int. Vet. Congr., London, Part 3, p. 705.
- Beardwell, C.G. (1964), Proc. R. Soc. Med. 57, 332.
- Bedarida, G., Bernasconi, C. and Pollini, G. (1961), Haematologica, 46, 1085.
- Bell, D.J., Bird, T.P. and McIndoe, W.M. (1965), Comp. Biochem. Physiol. 14, 83.
- Bell, F.N. and Irwin, M.R. (1938), J. infect. Dis. 63, 251.
- Bellis, C.J. and Snow, H.L. (1950), Ann. west. Med. Surg. 4, 223.
- Benacerraf, B., Biozzi, G., Halpern, B.N. and Stiffel, C. (1957), In 'Physiopathology of Reticulo-endothelial System, eds. Halpern, B.N. Benacerraf, B. and Delafresnaye, J.F. Blackwell Sci. Pub. Oxford, p. 52.
- Benacerraf, B., Thorbecke, G.J. and Jacoby, D. (1959), Proc. Soc. exp. Biol. Med. 100, 796.
- Benedict, A.A. (1965), Nature, Lond. 206, 1368.
- Bennett, I.L. and Cluff, L.E. (1957), Pharmac. Rev. 9, 427
- Beutler, E. (1959), Blood, 14, 103.
- Berczi, I., Bertok, L. and Bereznay, T. (1966), Can. J. Microbiol. 12, 1070.
- Bergner, P.E. (1965), Nature, Lond. 205, 975.
- Berlin, R.D. and Wood, W.B. (1964), J. exp. Med. 119, 697.
- Berman, S.E., Braun, K. and Rachmilewitz, M. (1945), Acta med. orient. (Tel-Aviv), 4, 175.
- Biozzi, G. and Stiffel, C. (1962), In 'Mechanisms of Cell and Tissue Damage Produced by Immune Reactions', 2nd Int. Symp. on Immunopathology, 1961. eds. Grabar, P. and Miescher, P. Benno-Schwabe, Basel, p. 249.

- Biozzi, G. (1968), Personal Communication.
- Blahd, W.H. (1962), In 'Mechanisms of Anaemia'. eds. Weinstein, I.M. and Beutler, E. McGraw-Hill, N.Y. p. 117.
- Boggs, D.R., Cartwright, G.E. and Wintrobe, M.M. (1966), Am. J. Physiol. 211, 51.
- Boggs, D.R., Chervenick, P.A., Marsh, J.C., Cartwright, G.E. and Wintrobe, M.M. (1968), J. Lab. clin. Med. 72, 177.
- Bokkenheuser, V. and Koornhof, H.J. (1959), Nature, Lond. 184, 109.
- Bokkenheuser, V. and Neter, E. (1962), Can. J. Microbiol. 8, 609.
- Bolton, J.H. (1949), Blood, 4, 172.
- Bowman, H.S., Brason, F.W., Mohn, J.F. and Lambert, R.M. (1961), Br. J. Haemat. 7, 130.
- Box, E.D. and Briggs, N.T. (1961), J. Immun. 87, 485.
- Boyden, S.V. (1950), Proc. Soc. exp. Biol. Med. 73, 289.
- Boyden, S.V. (1953), Nature, Lond. 171, 402.
- Boyden, S.V. and Grabar, P. (1954), Annls. Inst. Pasteur, Paris, 87, 257.
- Bradley, J. and Oppenheim, J.J. (1967), Clin. exp. Immunol. 2, 549.
- Braude, A.I. (1959), Trans. Am. Acad. Ophthal. Oto-lar. 63, 634.
- Braude, A.I., Carey, F.J., and Zalesky, M. (1955), J. clin. Invest. 34, 858.
- Braude, A.I., Carey, F.J., Sutherland, D. and Zalesky, M. (1955), J. clin. Invest. 34, 850.
- Braude, A.I., Carey, F.J. and Zalesky, M. (1955), J. clin. Invest. 34, 923.
- Brecher, G. (1967), Am. J. clin. Path. 47, 85.
- Britton, S. (1969), Immunology, 16, 527.
- Britton, /

- Britton, S. and Möller, G. (1965), In 'Immunity and Tolerance to a Lipopolysaccharide Antigen', *Czek. Acad. Sci. Prague*, p. 213.
- Britton, S. and Möller, G. (1968), *J. Immun.* 100, 1326.
- Britton, S., Wepsic, N. and Möller, G. (1968), *Immunology*, 14, 491.
- Brown, H.C., (1933), *Br. J. exp. Path.* 14, 413.
- Brown, G.M. (1950), *Can. Med. Ass. J.* 62, 472.
- Brown, G.M., Hayward, O.C., Powell, E.O. and Witts, L.J. (1944), *J. Path. Bact.* 56, 81.
- Buckland, F.E. (1960), *J. Hyg., Camb.* 58, 291.
- Burton, M.S. and Mollison, P.L. (1968), *Immunology*, 14, 861.
- Buxton, A. (1952), Ph.D. Thesis, Univ. of Cambridge.
- Buxton, A. (1957), 'Salmonellosis in Animals, Commonwealth Bur. of Animal Health, Rev. Ser. No. 5.
- Buxton, A. (1959 a), *Immunology*, 2, 203.
- Buxton, A. (1959 b), In 'Infectious Diseases of Animals' Diseases due to Bacteria, Vol. 2, eds. Stableforth, A.W. and Galloway, I.A., Butterworths, London, p. 481.
- Buxton, A. (1960), *J. comp. Path. Ther.* 70, 308.
- Buxton, A. and Allan, D. (1963), *Immunology*, 6, 520.
- Buxton, A. and Davies, J.M. (1963), *Immunology*, 6, 530.
- Calder, R.M., Steen, C. and Baker, L. (1939), *J. Am. med. Ass.* 112, 1893.
- Callender, S.T., Nickel, J.F., Moore, C.V. and Powell, E.O. (1949), *J. Lab. clin. Med.* 34, 90.
- Cameron, C., Graham, F., Dunsford, I., Sickles, G., MacPherson, C.R., Cahan, A., Sanger, R. and Race, R.R. (1959), *Br. med. J.* 2, 2
- Cameron/

- Cameron, J.A., Holtman, D.F. and Jefferies, C.D. (1960), J. infect. Dis. 106, 159.
- Cameron, R. and Rettger, L. (1934), J. Bact. 27, 86.
- Carey, F.J., Braude, A.I. and Zalesky, M. (1958), J. clin. Invest. 37, 441.
- Carstairs, K. (1968), Proc. R. Soc. Med. 61, 1309.
- Cartwright, G.E. (1966), Semin. Haemat. 3, 351.
- Cartwright, G.E. Lauritsen, M.A., Jones, P.J., Merrill, I.M. and Wintrobe, M.M. (1946), Science, N.Y. 103, 72.
- Cartwright, G.E. and Wintrobe, M.M. (1952), Adv. internal Med. 5, 165.
- Ceppellini, R. and De Gregorio, M. (1953), Boll. Ist. sieroter. milan., 32, 429.
- Chedid, L., Skarnes, R.C. and Parant, M. (1963), J. exp. Med. 117, 561.
- Chervenick, P.A., Boggs, D.R., Marsh, J.C., Cartwright, G.E. and Wintrobe, M.M. (1967), Proc. Soc. exp. Biol. Med. 126, 891.
- Cline, N.J. and Berlin, N.I. (1963), Blood, 21, 63.
- Chorpenning, F.W. and Hayes, J.C. (1959), Vox Sang. 4, 210.
- Cohen, F., Zuelzer, W.W. and Evans, M.M. (1960), Blood, 15, 884.
- Cohen, R.R. (1967), Poult. Sci. 46, 214.
- Cole, J.R. and Boyd, F.M. (1965), Poult. Sci. 44, 1551.
- Connor, D.G. and Kass, E.H. (1961), Nature, Lond. 190, 453.
- Cook, F.W. and Dearstyne, R.S. (1934), N. Carol. agric. Exp. Stn Tech. Bull. 44, 26.
- Cook, G.M.W., Heard, D.H. and Seaman, G.F. (1961), Nature, Lond. 191, 44.
- Coons/

- Coons, A.H. and Kaplan, M.H. (1950), *J. exp. Med.* 91, 1.
- Cooper, G.H. and Stuart, A.E. (1961), *Nature, Lond.* 191, 294.
- Conway, H. (1953), *J. clin. Path.* 6, 208.
- Coutes, V. and Marsh, B.E. (1966), *Poult. Sci.* 45, 1302.
- Cox, H.W. (1966), *Milit. Med.* 131, (Suppl.), 1195.
- Craddock, G.Jr. (1962), In 'Mechanisms of Anaemia', eds. Weinstein, I.M. and Beutler, E., McGraw-Hill, N.Y. Chap. 8.
- Creger, W.P., Tulley, E.H. and Hansen, D.G. (1956), *J. Lab. clin. Med.* 47, 686.
- Cremer, N. and Watson, D.W. (1957), *Proc. Soc. exp. Biol. Med.* 95, 510.
- Croft, J.D., Swisher, S.N., Gilliland, B.C., Bakemeirer, R.F., Leddy, J.P. and Weed, R.I. (1968), *Ann. intern. Med.* 68, 176.
- Crome, P. and Mollison, P.L. (1964), *Br. J. Haemat.* 10, 137.
- Curtice, C. (1902), *Rh. Isl. agric. Exp. Stn Bull.* 87, 10.
- Cutbush, M. and Mollison, P.L. (1958), *Br. J. Haemat.* 4, 115.
- Cutler, J.K. (1961), *J. Immun.* 86, 73.
- Dacie, J.V. (1950), *Nature, Lond.* 166, 36.
- Dacie, J.V. (1963), 'The Haemolytic Anaemias', Part II, The Auto-immune Haemolytic anaemias, 2nd Ed. J. and A. Churchill, London.
- Dacie, J.V. (1967), 'The Haemolytic Anaemias', Part III, Secondary or Symptomatic Haemolytic Anaemias, 2nd Ed. J. and A. Churchill, London.
- Dacie, J.V. (1968), *Proc. R. Soc. Med.* 61, 1307.
- Dacie/

- Dacie, J.V. and Lewis, S.M. (1968), 'Practical Haematology', 4th Ed. J. and A. Churchill, London.
- Dacie, J.V., Crookston, J.H. and Christenson, W.N. (1957), Br. J. Haemat. 3, 77.
- Dausset, J., Moullec, J. and Bernard, J. (1959), Blood. 14, 1079.
- Davidson, L.S.P. and Fullerton, H.W. (1938), Q.Jl. Med. N.S. 7, 43.
- Davies, D.A.L., Crumpton, M.J., MacPherson, I.A. and Hutchison, A.M. (1958) Immunology, 1, 157.
- Davies, D.F. (1958), Clin. Sci. 17, 563.
- De, S.N., Sengupta, K.P. and Chanda, N.N. (1954), Lancet, i, 807.
- De Gregorio, M. (1955), Boll. Ist. sieroter. milan., 34, 118.
- De Gregorio, M. and Di Nardo, A. (1955), Boll. Ist. sieroter. milan., 34, 123.
- De Gruchy, G.C. and Grimes, A.J. (1968), In 'Recent Advances in Clin. Path.' Series 5, ed. Dyke, S.C., J. and A. Churchill, London, p. 225.
- Dennis, E.W. and Saigh, A.S. (1945), Science, N.Y. 102, 280.
- Deutsch, H.F. and Morton, J.I. (1957), Science N.Y. 125, 600.
- Diengdoh, J.V. and Turk, J.L. (1968), Int. Archs. Allergy, appl. Immun. 34, 297.
- Diesem, C.D., Venzke, W.G. and Moore, E.N. (1958), Am. J. vet. Res. 19, 719.
- Dinning, J.S. (1962), Physiol. Rev. 42, 169.
- Domert, A.R. and Dimopoulos, G.T. (1965), Am. J. vet. Res. 26, 1047.
- Domert, A.R. and Dimopoulos, G.T. (1966), Am. J. vet. Res. 27, 1211.
- Domert, A.R. and Dimopoulos, G.T. (1967), Am. J. vet. Res. 28, 1173.
- Donahue, D.M., Motulsky, A.G., Giblett, E.R., Pirzio-Biroli, G., Viranuvatti, V. and Finch, C.A. (1955), Br. J. Haemat. 1, 249
- Dornhorst, /

- Dornhorst, A.C. (1957), Blood, 6, 1284.
- Dreesman, G., Larson, C.H., Pinckard, R.N., Groydon, R.M. and Benedict, A.A. (1965) Proc. Soc. exp. Biol. Med. 118, 292.
- Dresser, D.W. and Mitchison, N.A. (1968), Adv. Immun. 8, 129.
- Duffus, W.P.H. and Allan, D. (1968), Immunology, 15, 653.
- Ebaugh, F.G. Jr. Emerson, C.P. and Ross, J.F. (1953), J. clin. Invest. 32, 1260.
- Eddington, C.L. and Kampschmidt, R.F. (1968), Proc. Soc. exp. Biol. Med. 129, 580.
- Editorial, (1967), J. Am. med. Ass. 199, 999.
- Eisen, H. N. and Siskind, G.W. (1964), Biochemistry, 3, 996.
- Ejby-Poulsen, P. (1954), Nature, Lond. 174, 929.
- Epp, M. (1962), Immunology, 5, 287.
- Evans, R.S., Bingham, M., Hickey, M. and Hassett, D. (1959), Trans. Ass. Am. Physns. 72, 188.
- Evans, R.S., Bingham, M. and Weiser, R.S. (1963,a), J. exp. Med. 117, 647.
- Evans, R.S., Bingham, M. and Weiser, R.S. (1963,b), J. Lab. clin. Med. 62, 559.
- Farr, R.S. and Le Quire, V.S. (1950), Proc. Soc. exp. Biol. Med. 75, 661.
- Farrar, W.E. Jr. (1965), Proc. Soc. exp. Biol. Med. 118, 218.
- Farrar, W.E. Jr. and Corwin, (1966), Ann. N.Y. Acad. Sci. 133, 668.
- Farrar, W.E. Jr. and Magnani, T.J. (1964), Proc. Soc. exp. Biol. Med. 115, 596.
- Ferguson, /

- Ferguson, L.C., Irwin, M.R. and Beach, B.A. (1945), *J. infect. Dis.* 76, 31.
- Finch, C.A., Gibson, J.G., Peacock, W.C. and Fluharty, R.G. (1949), *Blood*, 4, 905.
- Findlay, G.M. and Brown, H.C. (1934), *Br. J. exp. Path.* 15, 148.
- Fine, M., Flocks, M., Feichtmeir, T.V.P. (1955), *Am. J. med. Sci.* 229, 670.
- Fisher, J.A. (1947), *Q. Jl. Med. N.S.* 16, 245.
- Flandin, C, Bernard, J. and Mallarmé, J. (1935), *Bull. Soc. méd. Hôp. Paris*, 51, 898.
- Flodin, P. and Killander, J. (1962) *Biochim. biophys. Acta.* 63, 403.
- Formal, S.B., Noyes, H.E. and Schneider, H. (1960), *Proc. Soc. exp. Biol. Med.* 103, 415.
- Fruhman, G.J. (1966), *Blood*, 27, 363.
- Fulthorpe, A.J. (1954), *J. Path. Bact.* 68, 315.
- Gear, J. (1955-1956), *Acta med. scandinav.* ¹⁵²2 (suppl. 306), p. 39.
- Gell, P.G.H. and Coombs, R.R.A. (1968), *'Clinical Aspects of Immunology'*, 2nd Ed. Blackwell Sci. Pub. Oxford.
- Gilbert, R.P. (1960), *Physiol. Rev.* 40, 245.
- Giles, C.M., Mourant, A.E., Parkin, D.M., Horley, J.F. and Tapson, K.J. (1959), *Br. med. J.* 2, 32.
- Gitlin, D., Landing, B.H. and Whipple, A. (1953), *J. exp. Med.* 97, 163.
- Golub, S., Gröschel, D. and Nowotny, A. (1967), *Bact. Proc.* 79.
- Golub, S., Gröschel, D and Nowotny, A. (1968), *J. R. E. Soc.* 5, 324.
- Goodman/

- Goodman, H.C. and Bozicevich, J. (1964), In 'Immunological Methods',
A Symposium organised by the Council for Inter. Organs. of
Med. Sciences, ed. Ackroyd, J.F., Blackwell Sci. Pub.
Oxford, p. 93.
- Good, R.A. and Thomas, L. (1952), J. exp. Med. 96, 625.
- Gordeuk, S., Glantz, J.P., Callenbach, E.W. and Thorpe, W.T. (1949),
Poult. Sci. 28, 385.
- Gordon, R.F. and Buxton, A. (1946), Vet. J., 102, 187.
- Gordon, R.F., Garside, J.S. and Tucker, J.F. (1959), Vet. Rec. 71, 300.
- Gordon-Smith, C.E. (1951), Lancet. ii, 1020.
- Goudie, R.B., Horne, C.H. and Wilkinson, P.C. (1966), Lancet, ii, 1224.
- Grabar, J. (1959), Annls. Inst. Pasteur, Paris, 96, 577.
- Gramlich, F. and Müller, H.E. (1963) Acta haemat. 30, 153.
- Grant, R. (1950), Am. J. Physiol. 160, 285.
- Greenbury, C.L., Moore, D.H. and Nunn, L.A. (1963), Immunology, 6, 421.
- Greisman, S.E. (1960), J. exp. Med. 112, 257.
- Greisman, S.E., Hornick, R.B., Carozza, F.A. and Woodward, E. (1964),
J. clin. Invest. 43, 986.
- Greisman, S.E., Hornich, R.B., Wagner, H.N., Woodward, W.E. and
Woodward, T.E. (1969), J. clin. Invest. 48, 613.
- Grey, H.M. (1964), Immunology, 7, 82.
- Grey, H.M., Briggs, W. and Farr, R.S. (1961), J. clin. Invest. 40, 703.
- Grobbelaar, B.G. (1958), M.D. Thesis, Univ. Witwatersrand, S. Afr.
- Grubb, R. and Swahn, B. (1958), Acta path. microbiol. scand. 43, 305.
- Gupta, J.D. and Reed, C.E. (1968), J. Immun. 101, 308.
- Hall, W.J. (1965), In 'Diseases of Poultry', eds. Biester, H.E. and
Schwarte, L.H. 5th Ed., Iowa State Univ. Press, Ames, Iowa,
p. 329.

- Hall, W.J., Legenhausen, D.H. and McDonald, A.D. (1949), *Poult. Sci.* 28, 344.
- Hagan, W.A. and Bruner, D.W. (1961), 'Infectious Diseases of Domestic Animals', 4th Ed., Bailliere, Tindall, and Cox, London, p. 220.
- Halpern, B.N., Biozzi, G., Benacerraf, B. and Stiffel, C. (1957), *Am. J. Physiol.* 189, 520.
- Hanig, M. (1948), *Proc. Soc. exp. Biol. Med.* 68, 385.
- Harbourne, J.F. (1958), *Vet. Rec.* 70, 443.
- Harris, I.M., McAllister, J.M. and Pranker, I.A.J. (1958), *Br. J. Haemat.* 4, 97.
- Hartman, R.S. and Nungester, W.J. (1953), *Proc. Soc. exp. Biol. Med.* 84, 710.
- Harvey, D.G. and Carne, P. (1960), *J. comp. Path. Ther.* 70, 84.
- Häyry, P. and Saxén, L. (1965), *Nature, Lond.* 205, 1105.
- Heddleston, K.L., Reberts, P.A. and Ritchie, A.E. (1966), *J. Immun.* 96, 124.
- Heilman, D.H. (1964), In 'Bacterial Endotoxins', Eds. Landy, M. and Braun, W., Rutgers Univ. Press, New Brunswick, p. 610.
- Heilman, D.H. (1965), *Int. Archs Allergy, appl. Immun.* 26, 63.
- Heilman, D.H. (1968), *Int. Archs Allergy, appl. Immun.* 33, 501.
- Heilman, D.H. and Bast, R. Jr. (1964), *Proc. Soc. exp. Biol. Med.* 116, 1019.
- Heilman, D.H. and Bast, R. Jr. (1967), *J. Bact.* 93, 15.
- Heppner, G. Weiss, D.W. (1965), *J. Bact.* 90, 696.
- Herring, W.B., Herion, J.C., Walker, R.I. and Palmer, J.G. (1963), *J. clin. Invest.* 42, 79.
- Hevesy, /

- Hevesy, G. and Ottesen, J. (1945), *Nature*, Lond. 156, 534.
- Hill, W.C. and Rowley, D. (1967), *Aust. J. exp. Biol. med. Sci.* 45, 693.
- Hill, G.J. and Weiss, D.W. (1964), In 'Bacterial Endotoxins', Eds. Landy, M. and Braun, W., Rutgers Univ. Press, New Brunswick, p. 422.
- Hinshaw, L.B., Jordan, M.M. and Vick, J.A. (1961), *J. clin. Invest.* 40, 1631.
- Hjort, T. (1968), *Int. Archs. Allergy, appl. Immun.* 34, 376.
- Ho, M. and Kass, E.H. (1958), *Proc. Soc. exp. Biol. Med.* 97, 505.
- Hopps, H.C. and Engles, C.F. (1957), *Lab. Invest.* 6, 77.
- Horowitz, H.I., Javid, J. and Spaet, T.H. (1960), *J. Dis. Child.* 99, 757.
- Hughes-Jones, N.C. (1963), *Br. med. Bull.* 19, 171.
- Hughes-Jones, N.C. and Mollison, P.L. (1956), *Clin. Sci.* 15, 207.
- Hughes-Jones, N.C., Mollison, P.L. and Veall, N. (1957), *Br. J. Haemat.* 3, 125.
- Hungerford, T. (1951), 'Diseases of Poultry', 2nd Ed., Angus and Robertson, London, p. 223.
- Hunter, R.J. (1960), *Archs. Biochem.* 88, 308.
- Iseki, S., Onuki, E. and Kashiwagi, K. (1958), *Gunma J. med. Sci.* 7, 7.
- Isliker, H.C. (1957), *Adv. Protein Chem.* 12, 387.
- Isliker, H.C. (1964) In 'Immunological Methods'. A Symposium organised by the Council for International Organizations of Med. Sciences. ed. Ackroyd, J.F. Blackwell Sci. Pub. Oxford, p.140
- Jacob, H.S. (1966), *Med. Clins. N. Am.* 50, 1679.
- Jaffe, P. and Hopkins, J. (1961), *Poult. Sci.* 40, 604.
- Jandl, J.H. (1957), *J. clin. Invest.* 36, 904.
- Jandl, J.H. (1960), *J. Lab. clin. Med.* 55, 663.
- Jandl/

- Jandl, J.H. (1966), *Am. J. Med.* 41, 657.
- Jandl, J.H. and Kaplan, M.E. (1960), *J. clin. Invest.* 39, 1145.
- Jandl, J.H. and Simmons, R.L. (1957), *Br. J. Haemat.* 3, 19.
- Jandl, J.H. and Tomlinson, A.S. (1958), *J. clin. Invest.* 37, 1202.
- Jandl, J.H., Jacob, H.S. and Daland, G.A. (1961), *New Engl. J. Med.* 264, 1063.
- Jandl, J.H., Jones, A.R. and Castle, W.B. (1957), *J. clin. Invest.* 36, 1428.
- Jandl, J.H., Simmons, R.L. and Castle, W.B. (1961), *Blood*, 18, 133.
- Jandl, J.H., Files, N.M., Barnett, S.B. and MacDonald, R.A. (1965), *J. exp. Med.* 122, 299.
- Jankovic, B.D. (1959), *Acta haemat.* 22, 279.
- Jensen, W.N., Moreno, G.D., Bessis, M.C. (1965), *Blood*, 25, 933.
- Johnson, G.D., Holborrow, E.J. (1965), *Biblthca. haemat.* 23, 492.
- Jones, A.R. and Silver, S. (1958), *Blood*, 13, 763.
- Jordan, M.M. and Hinshaw, L.B. (1964), *Proc. Soc. exp. Biol. Med.* 115, 455.
- Joske, R.A., McAlister, J.M. and Prankerd, T.A.J. (1956), *Clin. Sci.* 15, 511.
- Julianelle, L.A. and Reimann, H.A. (1926), *J. exp. Med.* 43, 87.
- Karalnik, B.V. (1965), *Byull. eksp. Biol. Med.* 60, 81.
- Karalnik, B.V. (1967), *J. Hyg. Epidem. Microbiol. Immun.* 11, 115.
- Karush, F. (1959), In 'Mechanisms of Hypersensitivity', eds. Shaffer, J.H., Lo Grippo, G.A. and Chase, M.W., J. and A. Churchill, London, p. 19.
- Keene, W.R., Silberman, H.R. and Landy, M. (1961), *J. clin. Invest.* 40, 295.

- Keogh, E.V., North, E.A. and Warburton, M.F. (1948), *Nature, Lond.* 161, 687.
- Kessel, R.W. and Braun, W. (1965), *Aust. J. exp. Biol. med. Sci.* 43, 511.
- Kimball, H.R. and Wolff, S.M. (1967), *Proc. Soc. exp. Biol. Med.* 124, 269.
- Kimber, R.J., Lander, H. and Robson, H.N. (1965), *J. Lab. clin. Med.* 65, 951.
- King, M.K. and Wood, W.B. Jr. (1958), *J. exp. Med.* 107, 305.
- Knisely, M.H. (1951), *Postgrad. Med.* 10, 15.
- Knisely, M.H. and Bloch, E.H. (1942), *Anat. Rec. (Suppl.)* 82, 426.
- Knisely, M.H., Bloch, L.H., Eliot, T.S. and Warner, L. (1947), *Science, N.Y.* 106, 431.
- Kleeman, C.R. and Epstein, F.H. (1957), *Am. J. Med.* 23, 488.
- Klein, E. (1889), *Zentbl. Bakt. Parasit. Kde* 5, 689.
- Klímes, B. and Celěr, V. (1960), *Sb. čsl. Akad. zeměd. Ved. Vět. Med.* 5, 765.
- Koppisch, E. and Oliver-González, J. (1959), *Proc. Soc. exp. Biol. Med.* 100, 827.
- Kominos, Z.D. and Rosenthal, M.C. (1953), *J. Lab. clin. Med.* 41, 887.
- Kuida, H., Gilbert, R.P., Hinshaw, L.B., Brunson, J.G. and Visscher, M.B. (1961), *Am. J. Physiol.* 200, 1197.
- Lambe, D.W. and Holtman, D.F. (1960), *Bact. Proc.* p. 133.
- Landsteiner, K. and Miller, C.P. Jr. (1925), *J. exp. Med.* 42, 853.
- Landy, M. and Johnson, A.G. (1955), *Proc. Soc. exp. Biol. Med.* 90, 57.
- Landy, M. and Braun, W. (1964), 'Bacterial Endotoxins', Rutgers Univ. Press, New Brunswick, N.J.
- Landy, M., Johnson, A.G., Webster, M.E. and Sagin, J.F. (1955), *J. Immun.* 74, 466.

- Landy, M., Skarnes, R.C., Rosen, F.S., Trapani, R.-J. and Shear, M.J. (1957), *Proc. Soc. exp. Biol. Med.* 96, 744.
- Leddy, J.P. (1966), *Semin. Haemat.* 3, 48.
- Lee, C.L. and Davidsohn, I. (1965), *J. Immun.* 94, 423.
- Lee, R.E. and Vazquez, J.J. (1962), *Lab. Invest.* 11, 580.
- Levine, B.B. and Redmond, A.P. (1967), *Int. Archs. Allergy, appl. Immun.* 21, 594.
- Levy, E. and Ruebner, B.H. (1967), *Am. J. Path.* 51, 269.
- Levy, E. and Ruebner, B.H. (1968), *Am. J. Path.* 52, 97.
- Lewis, S.M., Szur, L. and Dacie, J.V. (1960), *Br. J. Haemat.* 6, 122.
- Ley, A.B., Harris, J.P., Brinkley, M., Liles, B., Jack, J.A. and Cahan, A. (1958), *Science, N.Y.* 127, 1118.
- Lindeboom, G.A. (1950), *Acta haemat.* 4, 343.
- Lindquist, K. and Bauer, D.C. (1966), *Immunochemistry*, 3, 373.
- Lindsey, E.S., Donaldson, G.W.K. and Woodruff, M.F.A. (1966), *Clin. exp. Immun.* 1, 85.
- Lombardo, G. and Tamburino, G. (1966), *Panminerva med.* 8, 79.
- Loutit, J.F. (1946), *Proc. R. Soc. Med.* 39, 757.
- Lowenthal, J. and Lamana, C. (1953), *Am. J. Hyg.* 57, 46.
- Lowy, P.H., Keighley, G., Borsook, H. and Graybiel, A. (1959), *Blood*, 14, 262.
- Lucas, A.M. and Jamroz, C. (1961), 'Atlas of Avian Haematology', U.S. Dept. Agric. Monograph 25, U.S. Dept. Agric. Washington.
- Lüderitz, O., Westphal, O., Sievers, K., Kröger, E., Neter, E. and Braun, O.H. (1958), *Biochem. Z.* 330, 34.
- Makinodan, T., Wolfe, H.R., Goodman, M. and Ruth, R. (1952), *J. Immun.* 68, 219.

Margaretten/

Margaretten, W., McKay, D.G. and Phillips, L.L. (1967), *Am. J. Path.*
Path. 51, 61.

Marsh, W.L., Jenkins, W.J. and Walther, W.W. (1959), *Br. med. J.* 2, 63.

Marvin, H.N. and Lucy, D.D. (1957), *Acta haemat.* 18, 239.

Mengel, C.E., Metz, E. and Yancey, W.S. (1967), *Archs. intern. Med.*
119, 287.

Menzies, D.W. (1947), In 'Diseases of Poultry', Blount, W.P. Bailliere,
Tindall and Cox, London, p. 405.

Matangkasombut, P. and Seastone, C.V. (1968), *J. Immun.* 100, 845.

Michael, J.G. (1966), *J. exp. Med.* 123, 205.

Middlebrook, G. and Dubos, R.J. (1948), *J. exp. Med.* 88, 521.

Miescher, P. (1957), In 'Physiopathology of Reticulo-endothelial System',
eds. Holpern, B.N., Benacerraf, B. and Delafresnaye, J.F.,
Blackwell Sci. Pub. Oxford, p. 147.

Mishra, J.M. (1959), *Br. med. J.* 2, 409.

Mitus, W.J. (1966), *Med. Clins. N. Am.* 50, 1703.

Mollison, P.L. (1947), *Clin. Sci.* 6, 137.

Mollison, P.L. (1959), *Br. med. Bull.* 15, 59.

Mollison, P.L. (1961), *Clin. Sci.* 21, 21.

Mollison, P.L. (1962), *Proc. R. Soc. Med.* 55, 915.

Mollison, P.L. (1962), In 'Mechanisms of Cell and Tissue Damage
Produced by Immune Reactions', 2nd Int. Symp. Immunopath.
1961, eds. Grabar, P. and Miescher, P., Benno-Schwabe, Basel,
p. 267.

Mollison, P.L. (1967), 'Blood Transfusion in Clinical Medicine', 4th
Ed. Blackwell Sci. Pub. Oxford.

Mollison, P.L. and Hughes-Jones, N.C. (1958), *Vox Sang.* 3, 243.

Mollison/

- Mollison, P.L. and Hughes-Jones, N.C. (1967), *Immunology*, 12, 63.
- Mollison, P.L. and Patterson, J.C.S. (1949), *J. clin. Path.* 2, 109.
- Mollison, P.L. and Veall, N. (1955), *Br. J. Haemat.* 1, 62
- Mollison, P.L., Crome, P., Hughes-Jones, N.C. and Rochna, E. (1965),
Br. J. Haemat. 11, 461.
- Moolten, S.E., Clarke, E., Glaser, B.F., Katz, E. and Miller, B.S.
(1953), *Am. J. Med.* 14, 294.
- Moore, V.A. (1895), U.S.A. Dept. Agr. Bur. Anim. Ind. 12th and 13th
Annual Reps.
- Mott, P.D. and Wolff, S.M. (1966), *J. clin. Invest.* 45, 372.
- Muirhead, E.E., Groves, M. and Bryan, S. (1954), *J. clin. Invest.* 33,
1700.
- Mulholland, J.H., Wolff, S.M., Jackson, A.L. and Landy, M. (1965),
J. clin. Invest. 44, 920.
- Muschel, L.H. and Osawa, E. (1959), *Proc. Soc. exp. Biol. Med.* 101,
614.
- McFadzean, A.J.S. and Choa, G.H. (1953), *Br. med. J.* 2, 360.
- McGhee, R.B. (1965), In 'Progress in Protozoology', *Proc. 2nd Int.*
Congr. Protozoology, London, p. 171.
- Nairn, R.C. (1969), 'Flourescent Protein Tracing', 3rd Ed. E. & S.
Livingstone, Edinburgh.
- Natt, M.P. and Herrick, C.A. (1952), *Poult. Sci.* 31, 735.
- Necheles, T.F., Weinstein, I.M. and Le Roy, G.V. (1953), *J. Lab. clin.*
Med. 42, 358.
- Nelson, D.S. and Boyden, S.V. (1963), *Immunology*, 6, 264.
- Neter, E. (1956), *Bact. Rev.* 20, 166.
- Neter, E. (1963), In 'Conference on Cell-bound Antibodies', eds. Amos,
B., and Koprowski, H., Wistar Inst. Press, p. 35.
- Neter/

- Neter, E., Gorzynski, E.A., Westphal, O., Lüderitz, O. and Klumpp, D.J. (1958), *Can. J. Microbiol.* 4, 371.
- Neter, E., Westphal, O. and Lüderitz, O. (1955), *Proc. Soc. exp. Biol. Med.* 88, 339.
- Neter, E., Westphal, O., Lüderitz, O., Gorzynski, E.A. and Eichenberger, E. (1956), *J. Immun.* 76, 377.
- Neter, E., Westphal, O., Lüderitz, O. and Gorzynski, E.A. (1956), *Ann. N.Y. Acad. Sci.* 66, 141.
- Neter, E., Westphal, O., Lüderitz, O., Gorzynski, E.A. (1955), *Fedn. Proc.* 14, 473.
- Neter, E., Zak, D.A., Zalewski, J.N. and Bertram, L.F. (1952), *Proc. Soc. exp. Biol. Med.* 80, 607.
- Neter, E., Zalewski, J.N. and Zak, D.A. (1953), *J. Immun.* 71, 145.
- Neva, F.A. and Morgan, H.R. (1950), *J. Lab. clin. Med.* 35, 911.
- Newbould, B.B. (1965), *Immunology*, 9, 613.
- Noll, H. and Braude, A.I. (1961), *J. clin. Invest.* 40, 1935.
- Nowotny, A. (1969), *Bact. Rev.* 33, 72.
- Noyes, W.D., Bothwell, T.H. and Finch, C.A. (1960), *Br. J. Haemat.* 6, 43.
- Noyes, H.E., McInturf, C.R. and Blahuta, G.J. (1959), *Proc. Soc. exp. Biol. Med.* 100, 65.
- Nungester, W.J. and Klein, L.F. (1937), *Proc. Soc. exp. Biol. Med.* 36, 315.
- Okonogi, T., Fukai, K., Mitsuhashi, S., Nagai, M. and Kawakami, M. (1959), *Jap. J. exp. Med.* 29, 71.
- Olitzki, L. (1959), *Acta med. orient.* 8, 103.
- Osbaldiston, G.W. (1968), *Veterinarian, Lond.* 5, 33.
- Ottesen, J. (1948), *Nature, Lond.* 162, 730.
- Pappenheimer/

- Pappenheimer, A.M., Thomson, W.P., Parker, D.D. and Smith, K.E. (1945)
Q. Jl. Med. 14, 75.
- Pauling, L., Pressman, D. and Grossberg, A.L. (1944), J. Am. chem.
Soc. 66, 784.
- Pavlovskis, O. and Springer, G.F. (1967), Fedn. Proc. 26, 801.
- Penhale, W.J. (1965), Ph.D. Thesis, Univ. of Liverpool.
- Petz, L.W. and Fudenberg, H.H. (1966), New Engl. J. Med. 274, 171.
- Ponder, E. and Ponder, R.V. (1955), J. exp. Biol. 32, 175.
- Prince, W.R. (1965), Diss. Abstr. 26, 590.
- Prince, W.R. (1966), Poult. Sci. 45, 1149.
- Prince, W.R. and Garren, H. (1949), Poult. Sci. 43, 1353.
- Prince, W.R. and Garren, H.W. (1966), Proc. 13th World Poultry Congr.
Kiev. p. 395.
- Rappaport, S.I., Tatter, D., Coeur-Barron, N. and Hjort, P. (1964),
New Engl. J. Med. 271, 80.
- Ravin, H.A., Rowley, D., Jenkin, C. and Fine, J. (1960), J. exp. Med.
112, 783.
- Read, R.C., Wilson, G.W. and Gardner, F.H. (1954), Am. J. med. Sci.
228, 40.
- Redmond, W.B. (1948), Science, N.Y. 107, 199.
- Reed, L.J. and Muench, H. (1938), Am. J. Hyg. 27, 493.
- Reepmaker, J. (1952), J. clin. Path. 5, 266.
- Retief, F.P. and Hofmeyr, N.G. (1965), S. Afr. med. J. 39, 96.
- Riedler, F. and Scheitlin, W. (1969), Br. med. J. 1, 753.
- Rifkind, R.A. (1965), Blood, 26, 433.
- Rifkind, R.A. (1966), Am. J. Med. 41, 711.
- Ritts, R.E., Young, E.J. and Arndt, W.F. (1964), In 'Bacterial
Endotoxins', Rutgers Univ. Press, New Brunswick, N.J. p. 311.

- Rodnan, G.P., Ebaugh, F.G. Jr. and Fox, M.R.S. (1957), *Blood*, 12, 355
- Rosenquist, C.L. and Gilden, R.V. (1963), *Biochim. biophys. Acta*, 78, 543.
- Rottino, A. and Angers, J.W. (1962), *Blood*, 20, 750.
- Rowley, D., Howard, J.G. and Jenkin, C.R. (1956), *Lancet*, i, 366.
- Rowley, D., Turner, K.J. (1964), *Immunology*, 7, 394.
- Ruggieri, P.A. (1961), *Delaware St. Med. J.* 33, 77.
- Rutenburg, S.H., Rutenburg, A.M., Smith, E. and Fine, T. (1966), *Ann. N.Y. Acad. Sci.* 133, 663.
- Rutenburg, S.H., Skarnes, R., Palmerio, C. and Fine, J. (1967), *Proc. Soc. exp. Biol. Med.* 125, 455.
- Sabine, J. (1964), *Br. J. Haemat.* 10, 477.
- Saifi, M.F. and Vaughan, J.M. (1944), *J. Path. Bact.* 56, 189.
- Saito, T., Konishi, K., Mitsui, T., Inaba, T. and Okumura, H. (1961), *Ann. Rep. Tuberc. Kanazawa Daigaku.*, K.K., 19, 1.
- Sandage, C., Brandt, N. and Birkeland, J.M. (1951), *J. infect. Dis.* 88, 9.
- Scheidegger, J.J. (1955), *Int. Archs. Allergy appl. Immun.* 7, 193.
- Seaman, G.V. and Uhlenbruck, G. (1962), *Biochim. biophys. Acta*, 64, 570.
- Sell, S. and Braude, A.I. (1961), *J. Immun.* 87, 119.
- Shaw, A.B. (1951), *Lancet*, ii, 813.
- Sheagren, J.N., Wolffe, S.M. and Shulman, N.R. (1967), *Am. J. Physiol.* 212, 884.
- Sherman, J.D. and Friedell, G.H. (1962), *Archs. intern. Med.* 109, 33.
- Sherman, P.H., Krumhaar, D., Tanaka, T., Bennet, L.R. and Maloney, J.V. (1966), *Ann. Surg.*, 164, 23.
- Shideler, A.M. (1960), *Lab. Invest.* 9, 435.
- Shultz/

- Shultz, D.R. and Becher, E.L. (1967), J. Immun. 98, 473.
- Shumway, C.N. (1958), J. Lab. clin. Med. 51, 240.
- Shumway, C.N. (1964), Nature, Lond. 203, 1081.
- Shumway, C.N., Bokkenheuser, V., Pollock, O. and Neter, E. (1963), J. Lab. clin. Med. 62, 600.
- Shumway, C.N. and Pollock, O. (1965), J. Lab. clin. Med. 65, 432.
- Sieburth, J. McN. (1957), J. Immun. 78, 380.
- Sieburth, J. McN. (1958), Am. J. vet. Res. 19, 729.
- Singer, K. and Fisher, B. (1952), Blood, 7, 1216.
- Skillman, R.K., Spurrier, W., Friedman, I.A. and Schwartz, S.O. (1955), Archs. intern. Med. 96, 51.
- Smith, A. (1964), In 'Bacterial Endotoxins', Rutgers Univ. Press, New Brunswick, N.J. p. 38.
- Smith, E.E., Rutenburg, S.H., Rutenburg, A.M. and Fine, J. (1963), Proc. Soc. exp. Biol. Med. 113, 781.
- Smith, R.T. and Thomas, L. (1956), J. exp. Med. 104, 217.
- Smith, W.H. (1955), J. comp. Path. Ther. 65, 37.
- Smith, W.H. (1956), J. Hyg., Camb. 54, 419.
- Smith, W.W., Alderman, I.M. and Cornfield, J. (1961), Am. J. Physiol. 201, 396.
- Smits, H.N. (1966), Tijdschr. Diergeneesk, 91, 851.
- Snedecor, G.W. and Cochran, W.C. (1967), 'Statistical Methods', 6th Ed. Iowa State Univ. Press, Ames. Iowa.
- Solomon, J.B. (1968), Immunology, 15, 197.
- Spaun, J. (1952), Acta path. microbiol. scand. 31, 462.
- Spink, W. W. and Anderson, D. (1954), J. clin. Invest. 33, 540.
- Springer, G.F. (1956), J. Immun. 76, 399.
- Springer, G.F. (1963), Bact. Rev. 27, 191.
- Springer, G.F. (1964), In 'Bacterial Endotoxins', Rutgers Univ. Press, New Brunswick, N.J. p. 350.

- Springer, G.F. (1967), In 'Cross-Reacting Antigens and Neoantigens', ed. Trentin, J.J., The Williams and Wilkins, Co. Baltimore, p. 29.
- Springer, G.F. and Ansell, N.J. (1962), Proc. 8th Int. Soc. Blood Transfusion, Tokyo, 1960, p. 219.
- Springer, G.F. and Horton, R.E. (1964), J. gen. Physiol. 47, 1229.
- Springer, G.F., Horton, R.E. and Forbes, M. (1959), Ann. N.Y. Acad. Sci. 78, 272.
- Springer, G.F., Horton, R.E. and Forbes, M. (1959), J. exp. Med. 110, 221.
- Springer, G.F., Wang, E.T., Nichols, J.H. and Shear, J.M. (1966), Ann. N.Y. Acad. Sci. 133, 566.
- Springer, G.F., Williamson, P. and Brandes, W.C. (1961), J. exp. Med. 113, 1077.
- Stetson, C.A. (1955), J. exp. Med. 101, 421.
- Stetson, C.A. (1961), Bull. N.Y. Acad. Med. 37, 486.
- Stewart, F.S. and McLoughlin, M. (1968), Immunology, 15, 13.
- Stohlman, F. Jr. (1961), Proc. Soc. exp. Biol. Med. 107, 884.
- Stohlman, F. Jr. and Schneiderman, M.A. (1956), J. Lab. clin. Med. 47, 72.
- Stratton, F. and Jones, A.R. (1955), J. Immun. 75, 423.
- Stratton, F. and Renton, P.H. (1959), Br. med. J. 2, 244.
- Suess, J., Limentani, D., Dameshek, W. and Dolloff, M.J. (1948), Blood, 3, 1290.
- Sundaram, K., Phondke, G.P. and Ambrose, E.J. (1967), Immunology, 12, 21.
- Suter, E. (1964), J. Immun. 92, 49.
- Suter/

- Suter, E. (1964), In 'Bacterial Endotoxins', Rutgers Univ. Press, New Brunswick, N.J. p. 435.
- Suter, E. and Kersanow, E.M. (1961), Immunology, 4, 354.
- Suter, E., Ullman, G.E. and Hoffman, R.G. (1958), Proc. Soc. exp. Biol. Med. 99, 167.
- Sutherland, D.A., Eisentraut, A.M. and McCall, M.S. (1963), Br. J. Haemat. 9, 68.
- Swisher, S.N. (1964), A. Rev. Med. 15, 1.
- Szenberg, A., Lind, P. and Clarke, K. (1965), Aust. J. exp. Biol. med. Sci. 43, 451.
- Talmage, D.W. (1959), In 'Mechanisms of Hypersensitivity', eds. Shaffer, J.H., Lo Grippo, G.A. and Chase, M.W., J. and A. Churchill, London, p.3.
- Taylor, J., Maltby, M.P. and Payne, J.M. (1958), J. Path. Bact. 76, 491.
- Taylor, J., Wilkins, M.P. and Payne, J.M. (1961), Br. J. exp. Path. 42, 43.
- Thomas, L. (1954), A. Rev. Physiol. 16, 467.
- Thomas, L. (1964), New Engl. J. Med. 270, 1157.
- Tikoff, G., Kuida, H. and Chiga, M. (1966), Am. J. Physiol. 210, 847.
- Trapani, R.J., Waradvekar, V.S., Landy, M. and Sheer, M.J. (1962), J. infec. Dis. 110, 135.
- Truscott, R.B. and Inniss, W.E. (1967), Avian Dis. 11, 595.
- Tsumita, T. and Ohashi, M. (1964), J. exp. Med. 119, 1017.
- Turner, K.J., Jenkin, C.R. and Rowley, D. (1963), Aust. J. exp. Biol. med. Sci. 42, 229.
- Ullman, /

- Ultman, J.E. and Gordon, C.S. (1965), *Blood*, 26, 49.
- Van Kampen, E.J. and Heerspink, W. (1961), *Clinica chim. Acta.* 6, 630.
- Vaughan, J.M. and Saifi, M.F. (1939), *J. Path. Bact.* 49, 69.
- Veall, N. and Vetter, T.S. (1958), 'Radioisotope Techniques in Clinical Research and Diagnosis', Butterworths, Co. London.
- Visscher, M.B. (1965), *Physiol. Physns.*, Wash. 3, 1.
- Wagner, H.N. Jr., Iio, M. and Hornick, R.B. (1963), *J. clin. Invest.* 42, 427.
- Walter, H., Brake, J.M. and Selby, F.W. (1965), *Expl. Cell. Res.* 37, 420.
- Ward, A.R. and Gallagher, B.A. (1920), 'Diseases of Domesticated Birds', Macmillan, Co. N.Y.
- Warner, N.L. (1962), *Aust. J. exp. Biol. med. Sci.* 40, 105.
- Wasastjerna, C., Dameshek, W. and Kominos, Z.D. (1954), *J. Lab. clin. Med.* 43, 98.
- Watson, D.W. and Kim, Y.B. (1963), *J. exp. Med.* 118, 425.
- Weed, R., Eber, J. and Rothstein, A. (1961), *J. clin. Invest.* 40, 130.
- Weiner, W. (1957), *Br. J. Haemat.* 3, 276.
- Weiss, L. (1962), *Am. J. Anat.* 113, 51.
- Weissmann, G. and Thomas, L. (1964), In 'Bacterial Endotoxins', Rutgers Univ. Press, New Brunswick, N.J. p. 602.
- Weinstein, I.M. and Beutler, E. (1962), 'Mechanisms of Anaemia', Symp. Univ. California Med. Extn. Series, Los Angeles, McCraw-Hill, Co. N.Y.
- Weinstein, I.M. and Le Roy, G.V. (1953), *J. Lab. clin. Med.* 42, 368.
- Wels, A., Schnappauf, H. and Horn, V. (1967), *Zentbl. Vet. Med.* 14A, 733.

- Whitaker, J.A., Zuelzer, W.F., Robinson, A.R. and Evans, M. (1959),
J. Lab. clin. Med. 54, 282.
- Wiener, E. and Levanon, D. (1968), Lab. Invest. 19, 584.
- Wilson, G.S. and Miles, A. (1964), 'Topley and Wilson's Principles of
Bacteriology and Immunity', 5th Edn. Edward Arnold, London.
- Wilson, J.E. (1946), Vet. Rec. 58, 269.
- Wilson, J.E. (1958), Agric. Rev., Lond. 3, 23, (Pt. 9).
- Wilson, J.E. (1961), Poult. Rev. 1, 31.
- Wintrobe, M.M. (1967), 'Clinical Haematology', 6th Edn. Henry Kimpton,
London.
- Wintrobe, M.M., Greenberg, G.R. and Cartwright, G.E. (1946), Trans.
Ass. Am. Physns. 59, 110.
- Wirth, D. and Kubasta, F. (1939), Folia haemat. Lpz. 62, 43.
- Wiznitzer, T., Better, N., Rachlin, W., Atkins, N., Frank, E. and
Fine, J. (1960), J. exp. Med. 112, 1157.
- Wood, W.B.Jr. (1958), New Engl. J. Med. 258, 1023.
- Worlledge, S.M. (1969), Semin. Haemat. 6, 181.
- Yoffey, J.M., Makin, G.S., Yates, A.K., Davis, C.J.F., Griffiths, D.A.
and Waring, I.S. (1963), Ann. N.Y. Acad. Sci. 113, 790.
- Young, V.M., Gillem, H.C. and Akeroyd, J.H. (1962), J. Pediat. 60, 172.
- Youngner, J.S. and Nungester, W.J. (1944), J. infect. Dis. 74, 247.
- Zinkham, W.H. and Diamond, L.K. (1952), Blood, 7, 592.
- Zuckerman, A. (1964), Expl. Parasit. 15, 138.
- Zweifach, B.W. and Janoff, A. (1965), A. Rev. Med. 16, 201.